Antibacterial Polymer Coatings

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Abstract

A series of poly(sulfone)s with quaternary ammonium groups and another series with aldehyde groups are synthesized and tested for biocidal activity against vegetative bacteria and spores, respectively. The polymers are sprayed onto substrates as coatings which are then exposed to aqueous suspensions of organisms. The coatings are inherently biocidal and do not release any agents into the environment. The coatings adhere well to both glass and CARC-coated coupons and they exhibit significant biotoxicity. The most effective quaternary ammonium polymers kills 99.9% of both gram negative and gram positive bacteria and the best aldehyde coating kills 81% of the spores on its surface.

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1 INTRODUCTION

Decontamination of property and critical infrastructure after an attack with biological weapons has been shown to be an overwhelming task [EPA, 2002; GAO, 2003; GAO, 2005]. In addition, while it may be apparent in some instances that contamination has occurred, in some cases contamination may go undetected for some time. With any contamination event, it would be helpful to have antimicrobial agents on surfaces prior to the event to reduce the consequences. Conventional disinfectants and antimicrobial agents are based on low molecular weight liquids or gases, so their use is restricted by their volatility and the toxicity of their residues. As an alternative, polymeric materials with antimicrobial activities may be able to overcome these restrictions. The primary goal of this project was to design a polymer that kills microorganisms on contact. In addition, we developed a method to apply the antimicrobial polymer to a variety of surfaces (fabric, plastic, metal, etc.) by spraying from a solution using a benign solvent. Antimicrobial performance targets for the coating were to address the broad spectrum of threats - from the least persistent agents such as viruses (e.g., smallpox) and vegetative bacteria (e.g., plague) to highly persistent agents such as bacterial spores (e.g., anthrax). In addition, the coating needed to be durable and to not release toxic agents into the environment.

As noted, spores are more persistent than viruses and vegetative bacteria. Generally, viruses and vegetative bacterial cells can survive for periods of hours to several months once established on inanimate surfaces [Kramer, et al., 2006]. Viruses are essentially vessels that consist of nucleic acids enclosed by a protein capsule (the capsid) [Watson and Crick, 1956]. Some viruses are additionally surrounded by a lipid envelope which is captured from the host cell membrane. Activity and replication are initiated only when triggered by attachment to susceptible cells, resulting in infection. Viruses can be persistent under favorable conditions; however, they are not highly resistant to environmental stresses and are easily degraded by many chemical agents [Bosch, et al, 2006]. Many bacteria can exist in two primary states, either vegetative or dormant. When vegetative bacterial cells become dormant, they transform into endospores (spores). Vegetative cells are enveloped by a membrane that consists of a phospholipid bilayer containing a matrix of proteins, carbohydrates and various compounds essential for the cell to sense and function with its environment. In the vegetative state, most bacterial cells are susceptible to environmental stresses and many chemical agents are effective at killing them [Sinclair, et al., 2008; Maillard, 2007]. Bacterial spores are considerably different. Once the spore is formed from a vegetative cell, the cell is encased in a multi-layered shell called the coat. While coats differ for various bacteria, they generally consist of a thick and heavily cross-linked peptidoglycan cortex which is encased by three protein layers (under, inner and outer coats). Many spores are also surrounded by another layer, the exosporium; which is a balloon-like membrane that is comprised largely of a paracrystalline protein basal layer and an outer nap of collagen-like fibrils [Henriques and Moran, 2007]. Hence, spore coats are considerably more complex and robust than the membrane of a vegetative cell. Spore coats are highly resistant to environmental or microbial degradation, allowing their bacterial cells to survive for several decades to eventually germinate and reproduce [Sinclair, et al., 2008; Durrheim, 2009].

Quaternary ammonium (QA) compounds are among the most widely used antibacterial agents for medical and public health applications. QA compounds have been shown to be effective against both gram negative and gram positive bacteria [*Tashiro*, 2001]. The lethal mode of action is generally accepted to proceed by (1) adsorption of the positively charged QA compound on the negatively charged cell surface, (2) disruption of the cell membrane by a lipophilic chain on the QA compound, and (3) diffusion through the membrane leading to cell death. Cationic polymers with QA groups generally exhibit higher antimicrobial activities than their corresponding low molecular weight monomers [*Ikeda and Tazuke*, 1983]. The higher activity is attributed to greater electrostatic attraction between the cell and polymer due to the greater charge density of the polymer.

To impart antimicrobial activity to treated surfaced, a QA compound with a trimethoxysilyl functionality was developed by Dow Corning (Dow Corning® 5700 Antimicrobial Agent) in 1977. The active ingredient in the Dow product is 3-(trimethoxysilyl)-propyldimethyloctadecyl ammonium chloride The trimethoxysilyl groups react with hydroxyl groups on surfaces such as glass and cotton to form covalent bonds that retain the QA compound at the surface and prevent it from dissolving in water. The trimethoxysilyl groups can also react with each other to form a highly stable crosslinked silane coating bound to treated surfaces. These coatings have been shown to impart biocidal activity to surfaces in many applications without the release of chemical agents into the surrounding environment [*Isquith et al*, 1972; *Isquith et al*, 1973; *Speier and Malek*, 1982; *Walters et al.*, 1973]. While these coatings were very effective as a fungicide and an antibacterial agent, they have been ineffective against spores.

There are many reports of polymers with biocidal molecules, such as furanones [*Read et al.*, 2001], azoles [*Moon et al.*, 2003], or hydroxyquinolines [*Kenawy*, 2001] tethered to them, however very few have mentioned any sporicidal activity. In addition, toxic or carcinogenic solvents were required in order to apply these polymers to surfaces.

Recent reports describe sporicidal coatings incorporating unbound biocidal agents, such as metal oxide particles [*Ramakrishna and Subramanian*, 2008], chlorine dioxide [*Leung et al.*, 2008], iodine compounds [*Ahmad, et al.*, 2008][*Singh, et al.*, 2007], silver nanoparticles [*Lee, et al.*, 2008], benzophenone [*Hong and Sun*, 2009], or QA compounds [*Ahlstrom and Edebo*, 1998] mixed with or encapsulated in a polymeric binder. These coatings differ from our work in that the activities of these coatings are dependant on the slow release and eventual depletion of the biocidal agents.

While QA compounds are effective against viruses such as smallpox (*Variola vera*) and vegetative bacteria such as plague (*Yersinia pestis*), they are decidedly less effective against highly persistent agents such as bacterial spores (e.g., *B. anthracis*, agent of anthrax). QA compounds are sporostatic (they inhibit spore outgrowth) but they are not sporicidal [*Russel*, 1990]. In order for the proposed polymer coatings to address the broad spectrum biological threat, an additional functionality was needed. Glutaraldehyde and formaldehyde are common sporicidal agents that we tethered to the proposed coating via an alkyl chain. The length of the alkyl chain tether was designed to allow enough mobility for the aldehyde moieties to bind to and penetrate the outer layers of spores while preventing them from leaching out of the coating and into the surrounding environment. This approach reasoned that the combination of the high

charge density of the QA groups and the toxicity of the tethered aldehydes should result in a highly effective biocidal polymer.

Ideally, the improved polymer coating would be designed to avoid all of the shortcomings described from the previous work. The new coating would be insoluble in water, yet easy to apply to surfaces by spraying with an alcohol or water/alcohol solution. It would combine the antibacterial activity and electrostatic attraction of a QA polymer, with the sporicidal activity of glutaraldehyde (and/or other aldehydes). The sporicidal agent (e.g., an aldehyde) would not be releasable into the environment because it would be covalently bound to the polymer coating.

In phase 1 of this project, a series of novel poly(sulfone)s with pendant QA groups was synthesized and evaluated for antibacterial activity. The activities of QA compounds have been found to be strongly dependent on the length of the alkyl chains attached to the QA group [*Kourai et al.*, 1980; *Nakagawa et al.*, 1983; *Nakagawa et al.*, 1984], so we produced QA pendants of varying chain lengths to assess the efficacy of QA groups tethered at different lengths. We also varied the density of QA groups in the polymers to study the affect of surface concentration on performance. The solubilities of these polymers were tuned by exchanging the anions present in order to prepare solutions in alcohol mixtures that were sprayed onto surfaces to create active coatings.

Phase 2 of this project addressed the incorporation of aldehyde functional groups into the polymer via covalent bonding to the polysulfones. The approach used was to combine the aldehyde functional groups with QA groups on the same polymers to achieve a cooperative effect. Many different aldehyde structures and attachment strategies were used in order to maximize their effectiveness while keeping the synthesis as simple as possible.

2 QUATERNARY AMMONIUM POLYMERS

2.1   Sample Preparation

2.1.1 Polymer Synthesis

The synthetic scheme for preparation of the polymers is shown in Figure 1. The parent tetramethyl poly(sulfone), TMPS, is prepared according to a modification of the method of *Wang et al.* [2002] by the condensation of 4,4'-difluorophenyl sulfone with tetramethyl bisphenol A, TMBPA. In order to drive the reaction to high molecular weights, the product water was removed by azeotropic distillation with toluene. The weight average molecular weight for the batch of TMPS used to make the coatings in this study was 132,000 with a polydispersity of 2.1.

The benzylic methyl groups on TMPS could be brominated using *N*-bromosuccinimide (NBS) as a source of bromine and benzoyl peroxide as an initiator. The fraction of methyl groups converted to bromomethyl groups was controlled by adjusting the amount of NBS used. The fraction of methyl groups converted to bromomethyl groups was determined by ^{1}H NMR spectroscopy. Figure 2 shows the relationship between the amount of NBS used and the number of bromomethyl groups formed for several different batches of Br-TMPS. In all cases, the reaction temperature was 85 °C, the reaction time was 3 hours, and 0.05 equivalents of benzoyl peroxide (based on NBS) were used. The degree of functionality (DF) is defined as the number of bromomethyl groups per polymer repeat unit and thus has a maximum possible value of
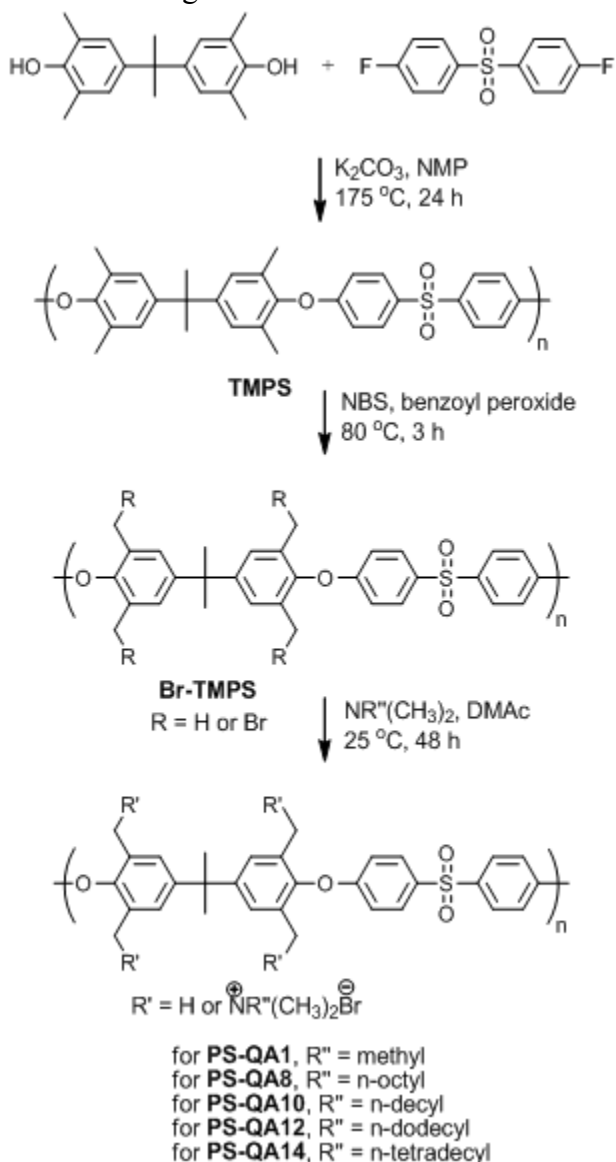
Figure 1. Synthesis of poly(sulfone) with quaternary ammonium groups.

4. In all cases, the DF achieved was approximately 60 % of the DF that would have resulted if every NBS molecule had reacted to make a bromomethyl group.

The PS-QA polymers prepared for this study were made from batches of Br-TMPS with DF values from 0.84 to 1.48. This range of values was chosen in order to study the effect of the density of QA groups without rendering any of the polymers soluble in water. The substitution reaction to form the quaternary ammonium groups took place at room temperature by mixing the desired tertiary amine with a DMAc solution of Br-TMPS. In all cases the neat amine was used, except for trimethylamine which was added as an aqueous solution.
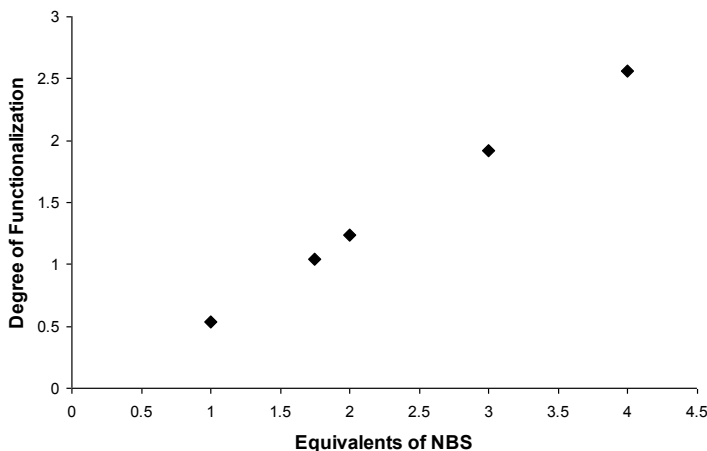
Figure 2. Efficiency of bromination in the synthesis of Br-TMPS. Equivalents of NBS are per polymer repeat unit. Degree of functionalization = number of bromomethyl groups per repeat unit.

When the QA groups were initially formed, the counter ions were bromide ions. Later, during work-up, the polymers were precipitated in 1 M HCl at which time the bromide ions were exchanged for chloride ions. In the chloride form, the PS-QA polymers were not very soluble in alcohols, so an excess of sodium laurate was added to help them dissolve. Exchange of the chloride ions for laurate ions caused the long, flexible chains of the laurate ions to become closely associated with the polymer chains. Although there was no covalent attachment of the laurate ions to the polymers, their presence enabled the PS-QA polymers to be dissolved in methanol, ethanol, or a mixture of both.

2.1.2 Application of Coatings

The polymers were dissolved in either ethanol or methanol and were then sprayed at a coverage of 5 mg polymer/in^2 onto stainless steel coupons previously painted with Chemical Agent Resistant Coating (CARC). The coupons had a surface area of 1.0 in^2. An airbrush attached to a compressed nitrogen source (40 psi) was used to spray the polymer solutions. After drying at 25 ^{o}C, the coupons were immersed in deionized water for 5 minutes to remove excess surfactant and then dried again at 25 ^{o}C prior to testing. The coated coupons were stored in the dark at room temperature for 24 to 72 hours prior to biological testing.
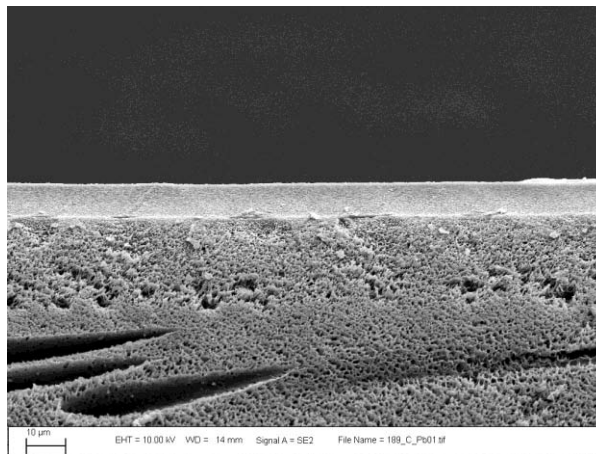
Figure 3. SEM image of cross section of an ultrafiltration membrane with PS-QA1 coating. Scale bar = 10 microns

To verify that the airbrushing process resulted in an even polymer coating, a poly(sulfone) ultrafiltration membrane was sprayed with one of the polymer solutions and dried. The membrane was then cross sectioned in a cryotome. Figure 3 shows an SEM image of the cross sectioned membrane with the polymer coating. A dense layer of PS-QA1 with a consistent thickness of nine microns can clearly be seen on the porous poly(sulfone) and this is taken as evidence that the coatings sprayed onto the stainless steel coupons are also of a relatively uniform thickness.

2.2   Biological Testing

2.2.1 Procedure

Coated coupons and control surfaces having no antibacterial polymeric coating were inoculated with liquid suspensions of vegetative bacteria including either gram positive *Bacillus globigii* or gram negative *Escherichia coli*. About 0.50 mL of the bacterial suspensions, with concentrations of about 10^7 CFU/mL, were applied directly to the coupon surfaces. After exposure times of 1, 4, and 6 hours, coated and control coupons (5 replicates of each) were immersed in a wash buffer solution and vortexed to release

bacteria. Samples were taken from the buffer solution and plated on Petri film for analysis.
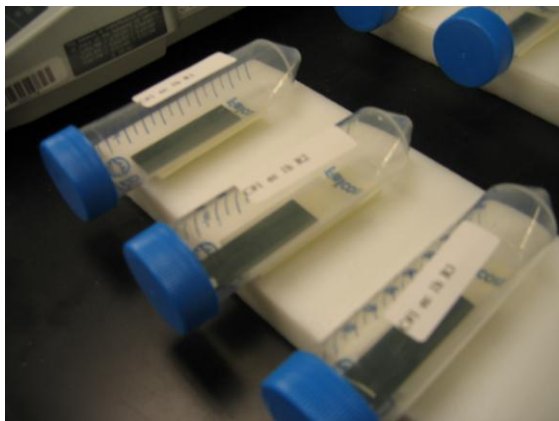
(a)
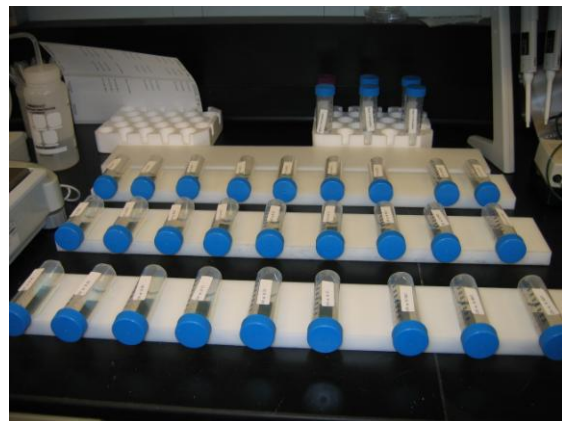
(b)
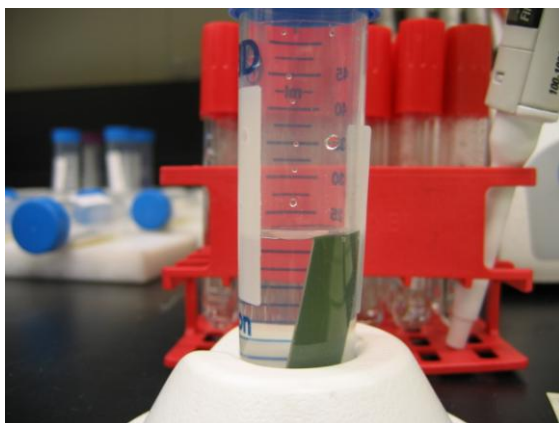
(c)

(d)

Figure 4. Bacterial testing of coatings: (a and b) Coupons inoculated with liquid suspensions of bacteria in sealed tubes. (c) Coupon in tube with wash buffer at end of exposure interval. (d) Samples plated on Petri film.

2.2.2 Results

Data from the biological tests are summarized in Table 1. The percent kill was calculated from the difference between the numbers of live colony forming units (CFUs) extracted from the control and coated coupons having equal exposure times on their surfaces. In all cases the gram positive *B. globigii* proved much more difficult to kill than the gram negative *E. coli*. This was unexpected, as it was contrary to other studies in which water soluble polycations showed greater biocidal activity against gram positive bacteria than against gram negative bacteria [*Ikeda and Tazuke*, 1984]. Surprisingly, the coating (PS-QA1) with the shortest alkyl chain (one carbon) was the most effective antimicrobial

coating against both organisms. This result was unexpected, since previous studies have indicated that QA compounds with longer alkyl chains (12 to 16 carbons) are more effective antibacterial agents than those with shorter chains [*Kourai et al.*, 1980; *Nakagawa et al.*, 1983; *Nakagawa et al.*, 1984]. It was observed that upon application of the aqueous suspensions of organisms to the samples, the liquid tended to spread out much more for the PS-QA1 coating than on the others. Thus, the effectiveness of this coating was likely due to its greater hydrophilicity, which resulted in more intimate contact between the bacteria and the QA groups. In other words, the PS-QA1 coating was the most hydrophilic, so aqueous samples spread out more, leading to more intimate surface contact for a more effective coating. In summary, the most hydrophilic QA coating showed very high activity against both of the vegetative bacteria tested.

Table 1. Biological testing data for five QA compound polymer coatings.

| Organism | Exposure time (h) | Percent of bacteria killed relative to CARC control | | | | |
| | | Number of carbons in alkyl chain in coating | | | | |
		1	8	10	12	14
E. coli (gram -)	1	99	13	0	65	0
	4	99	30	47	68	19
	6	99	55	82	76	15
B. globigii (gram +)	1	64	-11	13	5	-12
	4	97	-14	14	1	8
	6	99	-15	19	-24	9

Additional biological tests, with the results shown in Figure 5, were performed to study the effect of DF (Degree of Functionality) on bacterial kill. The DF value is defined as the number of QA groups per polymer repeat unit. Tests were performed on samples coated with PS-QA1 since it was the most promising candidate. As expected, the results showed that coatings with greater DF values were more effective against both bacteria. Note that the practical limit for the DF is about 1.48, as the PS-QA polymers become soluble in water at higher DF values and become unstable coatings.

(a)   (b)
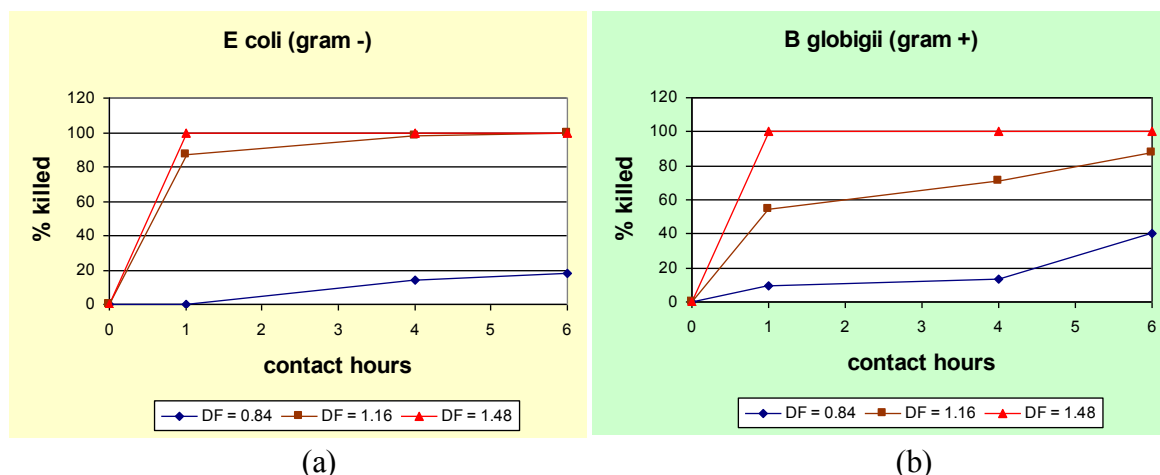
Figure 5. Percentage bacteria killed: of *E. coli* (a) and *B. globigii* (b) over time on PS-QA1 coatings with varying degrees of functionality (DF).

Two tests to evaluate the durability of the PS-QA1 (DF = 1.48) coating under simulated environmental conditions were performed. The first test (Figure 6) examined the effect of long-term exposure of the coating to deionized water on the bacterial kill efficacy. Sample coatings were immersed in deionized water at 25 °C and gently swirled for up to 14 days to see if coatings would wash off. Test results show that the effectiveness of the coating gradually decreased, but that after 14 days of water immersion, the coating still killed greater than 87 % of the *E. coli*. So even at a relatively low DF of 1.48, PS-QA1 was slightly soluble in water. This issue could be mitigated by adding a trialkoxy functional group to the polymer that could bond with surface groups, much like the scheme used in the Dow Corning® 5700 Antimicrobial Agent discussed in chapter 1. It should also be noted that most of the potential applications for an antibacterial coating such as this do not involve constant exposure to water, so these testing conditions represented an accelerated degradation process.

E coli (gram -)
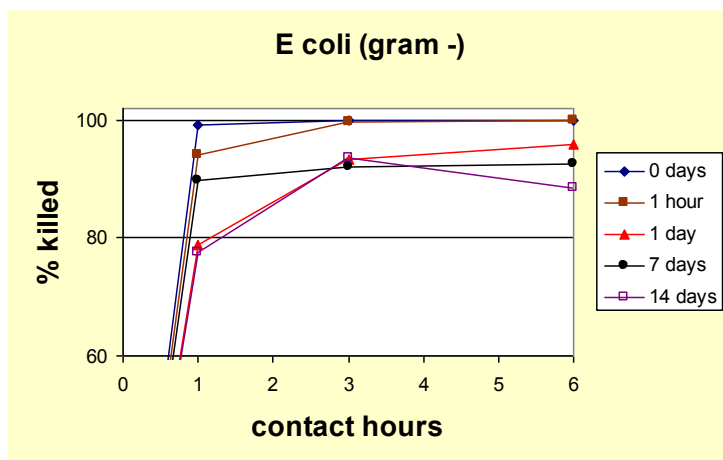
Figure 6. Percentages of *E. coli* killed on PS-QA1 coatings that have been exposed to water.

E coli (gram -)
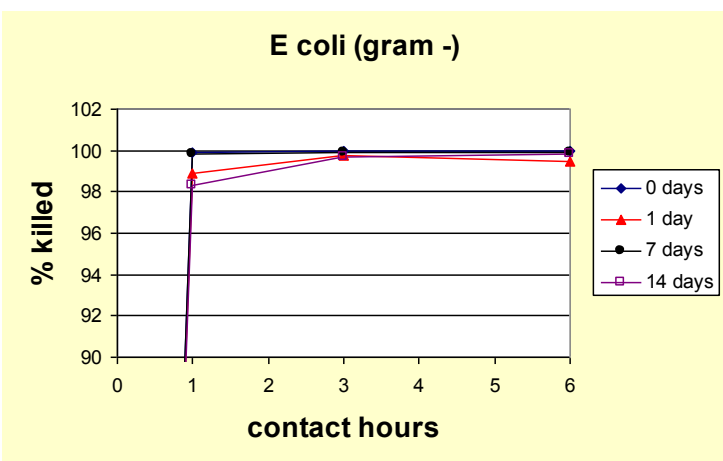
Figure 7. Percentages of *E. coli* killed on PS-QA1 coatings exposed to a diurnal temperature cycle.

In the second durability test, coated samples were placed in an oven with a simulated diurnal temperature cycle (117 °F maximum, 80 °F minimum) for 14 days. The relative humidity ranged from 7 to 17 % during the test. The results from this test (Figure 7) indicate that temperature cycling had virtually no effect on the ability of the coatings to kill bacteria. The slight fluctuations in the data in Figure 7 are within the error of the measurements, and are not likely to represent any real changes in the coatings.

2.3   Conclusions

The most hydrophilic QA coating showed very high activity against both of the vegetative bacteria tested.   Additional functionality will need to be incorporated to provide sporicidal capability.   The QA coatings could be applied easily to a variety of surfaces (fabric, plastics, building materials, etc.) by spraying, and could provide a passive first line of defense for personnel, property, and critical infrastructure against biological attacks.   This would directly support the DHS strategic goal of protection by safeguarding people, critical infrastructure, property, and the economy of our nation from acts of terrorism and other emergencies.

2.4   Experimental

2.4.1 Materials

4,4'-Isopropylidenebis(2,6-dimethylphenol) (tetramethyl bisphenol A or TMBPA) was recrystallized from toluene and dried under vacuum.   4-Fluorophenyl sulfone was recrystallized from ethanol and dried under vacuum.   All other reagents and solvents were purchased from commercial vendors and used without further purification.

Synthesis of Tetramethyl Poly(sulfone) (TMPS)
The parent polysulfone with four methyl groups on each repeat unit (TMPS) was prepared by a variation of the method for preparing a similar poly(sulfone) described by *Wang et al*. [2002].   TMBPA (8.53 g, 30 mmol), 4,4'-difluorophenyl sulfone (7.63 g, 30 mmol), K_2CO_3 (8.29 g, 60 mmol), anhydrous *N*-methylpyrrolidinone (NMP, 98 mL), and toluene (49 mL) were charged to a 3-necked flask equipped with a mechanical stirrer and a nitrogen flow passing into the flask and out through a distillation head.   The reaction was heated at 150 $^{\circ}$C for 4 h while toluene and water were removed as an azeotrope by distillation.   The temperature was raised to 160 $^{\circ}$C and held there for 19 h.   After cooling to room temperature, the reaction was poured into 0.1M HCl (0.5L) and mixed in a Waring blender.   The precipitate was Soxhlet extracted with water for 24 hours and then dried under vacuum to yield a tan solid (14.66 g, 98%).

Synthesis of brominated TMPS (Br-TMPS)
TMPS (32.0 g) was dissolved in 1,1,2,2-tetrachloroethane (470 mL) in a flask equipped with a magnetic stir bar and a condenser.   The solution was heated to 85 $^{\circ}$C and *N*-bromosuccinimide (20.0 g, 112 mmol) and benzoyl peroxide (1.36 g, 5.62 mmol) were added.   After 3 h, the reaction was cooled and poured into excess ethanol.   The precipitate was mixed with ethanol in a Waring blender.   The solid was then collected by filtration and triturated with water and then ethanol before being dried under vacuum at room temperature to yield a tan solid (37.01 g, 91%).

Synthesis of Poly(sulfone) with Benzytrimethyl Ammonium Groups (PS-QA1)
Br-TMPS (1.70 g) was dissolved in 42 mL dimethylacetamide.   A 45 wt. % aqueous solution of trimethylamine (1.0 mL, 6.5 mmol) was added and the solution was stirred at room temperature for 48 h.   The solution was poured into 1M HCl (200 mL), mixed, and filtered.   The precipitate was mixed with water in a Waring blender and then triturated

with more water before drying under vacuum at room temperature to yield a white solid (1.61 g, 92%).

The dry polymer was mixed with a solution of lauric acid in methanol (1 wt. %) to form a solution with a polymer concentration of 2 wt. %.

Synthesis of Poly(sulfone) with Benzydimethyl-n-octyl Ammonium Groups (PS-QA8)

This synthesis was the same as described for PS-QA1 except that dimethyl-n-octylamine was used instead of trimethylamine. The dry polymer was mixed with a solution of lauric acid (1 wt. %) in methanol to form a solution with a polymer concentration of 3 wt. %.

Synthesis of Poly(sulfone) with Benzydimethyl-n-decyl Ammonium Groups (PS-QA10)

This synthesis was the same as described for PS-QA1 except that dimethyl-n-decylamine was used instead of trimethylamine. The dry polymer was mixed with a solution of lauric acid (1 wt. %) in a mixture of methanol and ethanol (1:1 v/v) to form a solution with a polymer concentration of 2.5 wt. %.

Synthesis of Poly(sulfone) with Benzydimethyl-n-dodecyl Ammonium Groups (PS-QA12)

This synthesis was the same as described for PS-QA1 except that dimethyl-n-dodecylamine was used instead of trimethylamine. The dry polymer was mixed with a solution of lauric acid (1 wt. %) in ethanol to form a solution with a polymer concentration of 1.7 wt. %.

Synthesis of Poly(sulfone) with Benzydimethyl-n-tetradecyl Ammonium Groups (PS-QA14)

This synthesis was the same as described for PS-QA1 except that dimethyl-n-tetradecylamine was used instead of trimethylamine. The dry polymer was mixed with a solution of lauric acid (1 wt. %) in ethanol to form a solution with a polymer concentration of 2.0 wt. %.

2.4.2 Characterization and Measurements

Gel permeation chromatography (GPC) was performed with a liquid chromatograph equipped with a Viscotek VE2001 isocratic pump and autosampler and a Viscotek VE3580 refractive index detector.   The mobile phase was tetrahydrofuran and the system was operated at 25 °C with a flow rate of 1.0 mL min^{-1}. The weight-average molecular weights were measured by calibration with polystyrene standards.

^{1}H NMR spectra of the polymers were obtained on a Bruker spectrometer using 5 mm o.d. tubes. Sample concentrations were about 5 % (w/v) in CDCl$_3$ containing 1 % TMS as an internal reference.

2.4.3 Biological Testing Protocols

2.4.3.1 Vegetative Bacteria Tests

General test preparation
1. Label petrifilm.
2. Place 50-ml tubes on plastic risers with lose caps. Place the conicals on their sides and tape to board to prevent the tubes from rolling.
3. Locate five (5) polymeric coated coupons, five (5) blank (CARC) coupons in sterile 50 ml screw-top conical tubes, one coupon per tube.
4. Place five empty 50-ml tubes in an upright rack.
5. Repeat for each time frame.
6. Label tubes. (R1–R5 for each time frame 6h, 3h, & 1h for the coated coupons, C1-C5 for the CARC controls, and 1-5 for the no coupon controls)
7. Make 2 or more liters of sterile phosphate buffer solution (PBS).
8. Make 5 or more racks of DI water blanks and autoclave sterilize.
9. Make 250 ml of Nutrient Broth and autoclave sterilize.

Microorganism preparation
Prepare *E. coli* and *B. Atrophaeus* suspensions using the following procedure:
Only one organism is tested per day (unless there are two people for testing).

Done the afternoon before the test.
1. Place 30 ml of Nutrient Broth in labeled sterile 50 conical tube.
2. Take one bead from cryobank freeze storage (Blue tubes are E coli and Yellow tubes are E herbicola) and place in the nutrient broth. (This is the stock suspension.)
3. Vortex for 15 seconds.
4. Plate stock suspension to confirm concentration of ~10^7. Incubate at 26 degrees for 18 to 24 hours (overnight).

Done the day of the test.
5. Centrifuge stock suspension for five (5) minutes at 3000rpm.
6. Re-suspend pellet in 10 ml sterile phosphate buffer solution (PBS) by vortexing.
7. Centrifuge suspension for five (5) minutes at 3000rpm.
8. Re-suspend pellet in 10 ml sterile phosphate buffer solution (PBS) by vortexing.
9. Centrifuge suspension for five (5) minutes at 3000rpm.
10. Re-suspend pellet in 10 ml sterile phosphate buffer solution (PBS) by vortexing.
11. Determine organism concentration by plating.
12. Prepare serial dilutions 0-7 using DI water blanks. Plate tube #1 dilutions 4-6, tube #2 dilutions 3-5, tube #3 dilutions 2-4, tube #4 dilutions 1-3, tube #5 dilutions 0-2, tube #6 dilutions 0-1, and tube #7 dilutions 0

Surface inoculation
1. Locate five (5) polymeric coated coupons, five (5) blank (CARC) coupons in sterile 50-ml screw-top conical tubes and five empty 50-ml tubes for each time frame.

2. Inoculate each coupon with 500 µl of the prepared bacterial suspension ($\sim 10^5$ CFM per ml).   From original stock dilution tube number 2.
3. Place cap loosely on conical.
4. Inoculate the 6 hour contact tubes first, then the 4 hour tubes and finally the 1 hour tubes.
5. Start timer once both the polymeric coated and control coupons (including the 5 controls in the upright rack) for each time have been inoculated.

Microorganism Extraction and Analysis
At the end of the contact period:
1. Place conical upright in rack
2. Add 22 ml PBS buffer.
3. Seal with a cap.
4. Extract immediately by vortexing for 15 seconds.
5. Prepare serial dilutions of the extracted suspension (dilutions 1-4).
6. Plate 1.0 ml of the original suspension and each dilution to Petri film.
7. Incubate plates at 37°C in ambient air and examine after 24-36 hours.
8. Count colonies on the countable plates (30 to 300 CFU/ plate).
9. Calculate CFU (plate count x dilution factor x 30 = Total CFU per sample).
10. Enter counts into the spreadsheet.

2.4.3.2 Water Durability Study

General test preparation (best done the day before)
General test preparation will be the same as for normal tests. Refer to test protocol.

Microorganism preparation
Organism (*E. coli*) preparation will be the same as normal. Refer to test protocol.

Surface preparation and treatment:
1. UV sterilize glass containers for 15 minutes in Biological safety cabinet.
2. Set up shaker table and set at speed 45 (enough rotation to maintain liquid movement without sloshing over the edges).
3. Fill sterilized glass containers with 1100ml of sterile DI water.
4. Place 15 coated coupons in one container and 15 CARC coupons in a second container. There will be a total of 5 sets of coated coupons and 5 sets of CARC coupons. Ten glass dishes stacked on the shaker table.
5. Five tests will be conducted over a three week time line.
   a) Dry samples
   b) 1 hour H2O
   c) 24 hour H2O
   d) 1 week H2O
   e) 2 weeks H2O

6. See Durability studies calendar H2O sheet for specifics on which coupons were tested on which day.

7. At specified time the glass containers are removed from the shaker table and the coupons are removed with sterile tweezers.
8. The coupons are placed coating/CARC side up on a piece of paper towel and set in a hood to dry for a minimum of 4 hours. To accommodate the 6 hour test time the samples were left to dry overnight.
9. The glass dish had the water dumped out and then was placed empty back on the shaker table (as a place holder).
10. On test day the coupons are inoculated the same as a normal test.

Surface Inoculation
Surface inoculation will be the same as normal. Refer to test protocol.

Microorganism Extraction and Analysis
Organism extraction and analysis will be the same as normal. Refer to test protocol.

2.4.3.3 Heat Durability Study

General test preparation (best done the day before)
General test preparation will be the same as for normal tests. Refer to test protocol.

Microorganism preparation
Prepare *E. coli* and *E. herbicola* suspensions using the following procedure:
Organism preparation will be the same as normal. Refer to test protocol.

Surface preparation and treatment:
1. Set up Oven program to cycle between 32°C to 49°C to simulate night and day. The following program was used.

Step	1	2	3	4	5	6	7	8	Repeat	9	
Temp °C	32	28	28	32	38	49	49	38	30	32	End
Time hrs	2	2	6	2	1	1	6	4	Times	1	

2. Place 4 sets of coupons (one set =15 coated coupons and 15 CARC coupons) in the oven.

Four tests will be conducted over a three week time line.
      a. Not Heated
      b. 24 hour H2O
      c. 1 week H2O
      d. 2 weeks H2O

3. See Durability studies calendar Oven study sheet for specifics on which coupons were tested on which day.
4. On specified day remove coupons from oven and place in 50-ml conicals prepared for test.
5. On test day the coupons are inoculated the same as a normal test.

Surface Inoculation
Surface inoculation will be the same as normal. Refer to test protocol.

Microorganism Extraction and Analysis
Organism extraction and analysis will be the same as normal. Refer to test protocol.

3 ALDEHYDE POLYMERS

3.1 Uncharged Poly(sulfone)s with Tethered Aldehydes

As discussed in chapter 1, the goal of phase 2 of this project was to design polymer coatings with aldehyde functional groups so that they might be toxic to spores. The initial approach to this problem was to graft side chains onto a poly(sulfone) via Friedel-Crafts acylation (Figure 8). The reagent chosen for this reaction was methyl adipoyl chloride since the grafted side chain would have a terminal methyl ester which could then be reduced to an aldehyde. Unfortunately, this reaction failed to work after several attempts. Both AlCl$_3$ and ZnCl$_2$ were tried as the Lewis acid catalysts, but ^{1}H NMR analysis of the reaction products indicated that no grafting had occurred. This was unexpected since the poly(sulfone) used had a Radel®-type structure which has electron-rich activated sites on the biphenyl part of the repeat unit. However, since this reaction showed 0% conversion, it was abandoned in favor of another approach.
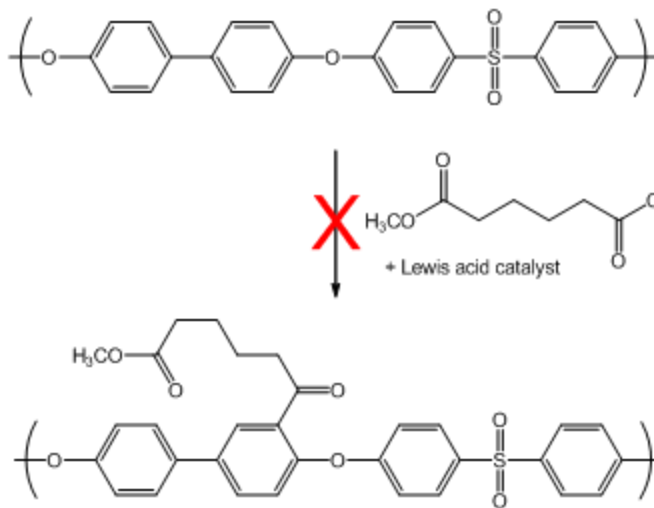
Figure 8. Synthetic scheme for attachment of side chains by Friedel-Crafts acylation.

The second approach to the attachment of aldehydes was to graft side chains onto the polymers via an ether linkage. To do this, a new poly(sulfone) with pendant methoxy groups was prepared under the same reaction conditions used to prepare TMPS except that the TMBPA was replaced with 2-methoxyhydroquinone as shown in Figure 9. The resulting poly(sulfone) could then be treated with boron tribromide to convert the methoxy groups into hydroxyl groups which could then serve as the points of attachment for the side chains. The side chains were attached through a Williamson ether synthesis by deprotonating the the hydroxyl groups on the polymer with a weak base (potassium carbonate) and then allowing an S$_N$2 displacement of the halogen atom on the side chain reagent. Side chains with terminal methyl esters and with terminal acetals (the R groups in Figure 9) were prepared and attached to the polymer. These groups were chosen because it was believed that they could be easily converted into aldehydes after they were attached to the polymers. However, attempts to reduce the methyl ester to an aldehyde using diisobutylaluminum hydride yielded only the ester starting material and so this approach was abandoned. The polymers with acetal side chains were subjected to hydrolysis conditions in a mixture of tetrahydrofuran and aqueous HCl and about half of the acetal groups were hydrolyzed to aldehydes. Unfortunately, these polymers were not soluble in the alcohol/water solvents that were stated in the objectives of the project. Nevertheless, one of these polymers (with a 6-carbon side chain, named PSE6) was sprayed from a chloroform solution. The performance of this

coating was very poor (see discussion in section 3.3) and this approach was also abandoned in favor of a new method (see section 3.2).
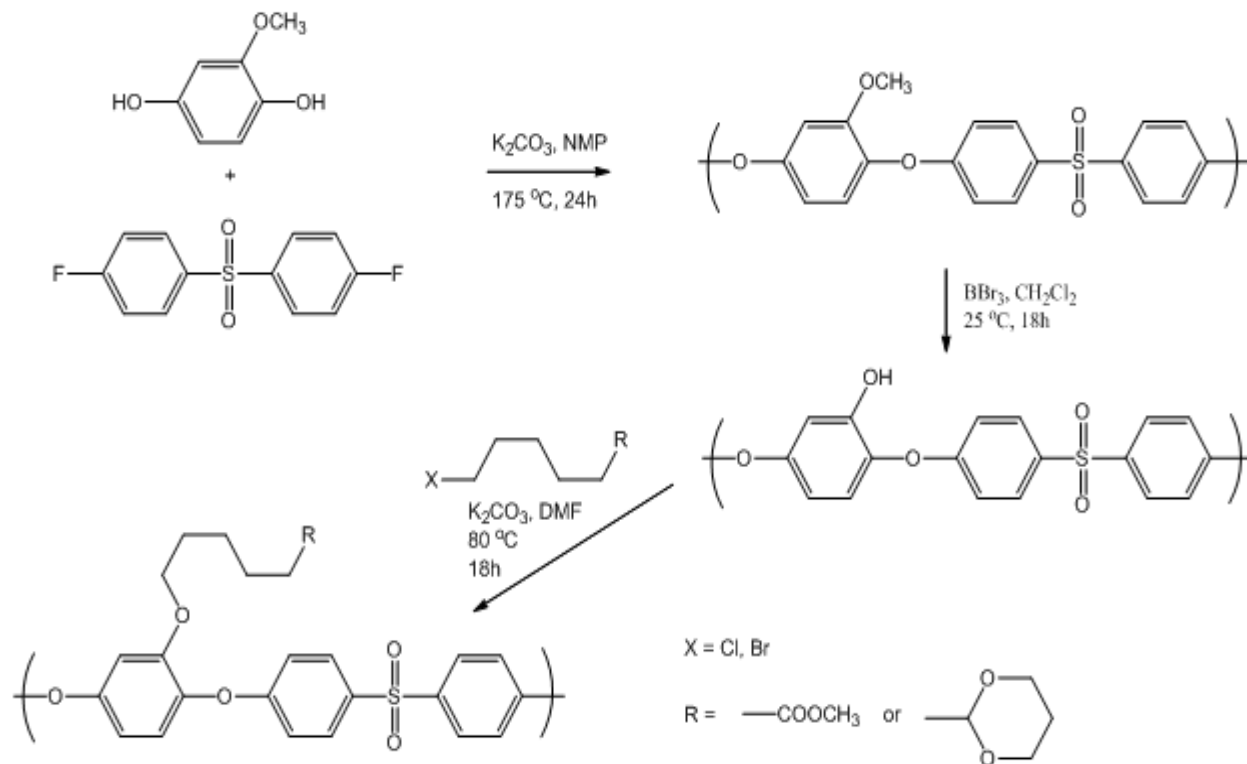
Figure 9. Synthetic scheme for attachment of side chains by ether linkages.

3.2 Poly(sulfone)s with Quaternary Ammonium and Aldehyde Functionalities

3.2.1 Scheme and Tertiary Amine Building Blocks

The third, and final approach to preparing poly(sulfone)s with aldehydes turned out to be the simplest in concept and the most useful. The approach is illustrated in Figure 10 and it is based on the same scheme used to make the PS-QA polymers (Figure 1). The same parent polymer, Br-TMPS, was used and it was again treated in solution with tertiary amines to make QA groups. The difference in this case was that instead of having just alkyl chains attached to the tertiary amines, the new approach used tertiary amines that had either aldehydes or protected aldehydes attached to them. This amounted to a kind of "click" chemistry since a variety of groups could be located on any given tertiary amine which could then attach itself to the polymer. Both trialkyl and pyridinyl type amines could be used and both are illustrated in Figure 10, where R represents an aldehyde or and aldehyde precursor as well as a spacer, in some cases.

A wide variety of tertiary amines were prepared for this approach and many others were considered but they were unable to be prepared or isolated. Figure 11 shows the

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structures that were commercially available, while Figure 12 illustrates the structures and the schemes for the tertiary amines that were synthesized. Structures and associated schemes for the tertiary amines that could not be synthesized and/or isolated are presented in Figure 13.
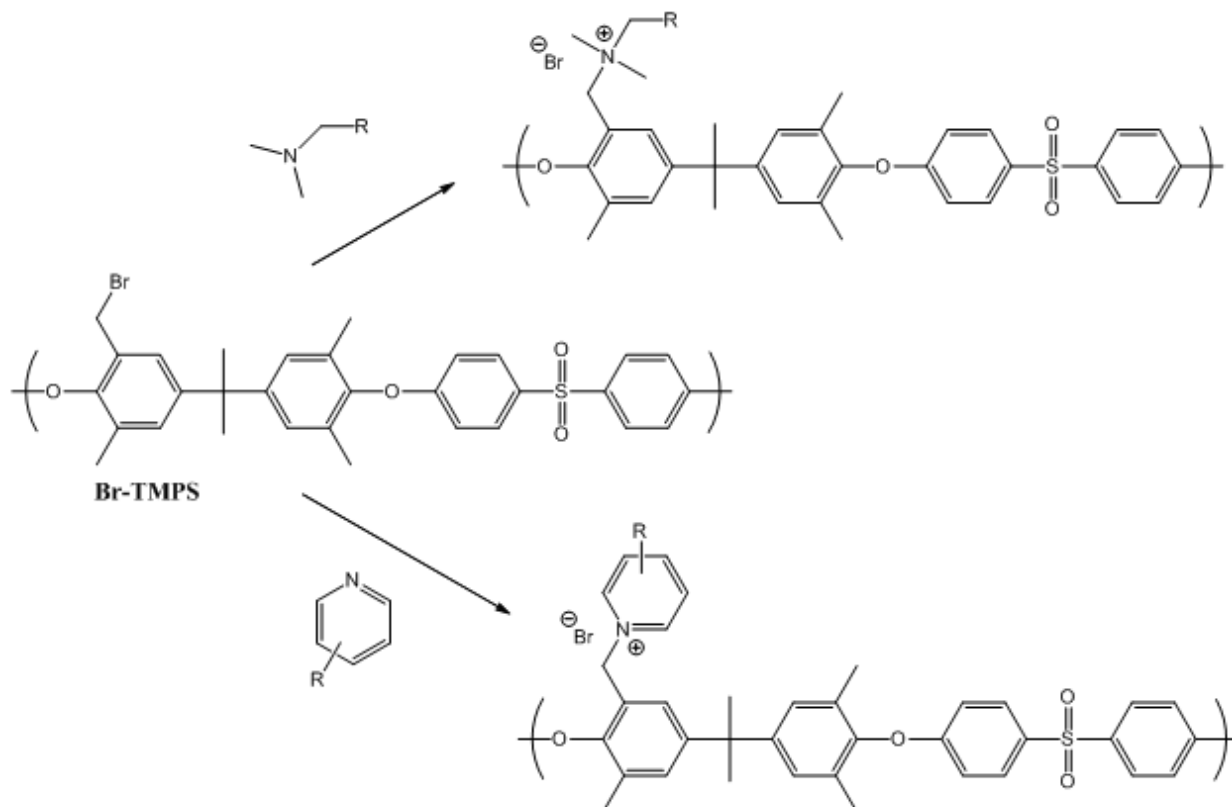
Figure 10. "Click" chemistry scheme for attaching aldehydes or aldehyde precursors via the formation of QA groups.
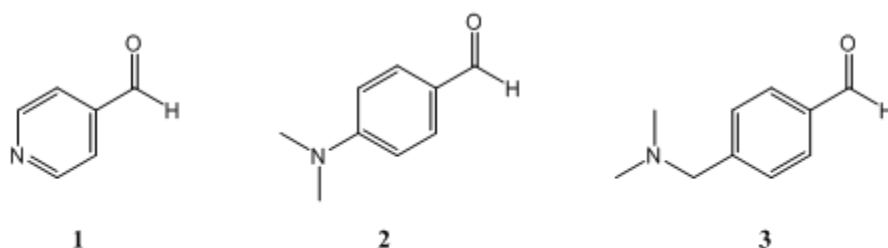
Figure 11. Commercially available tertiary amines for "click" chemistry synthesis.

28
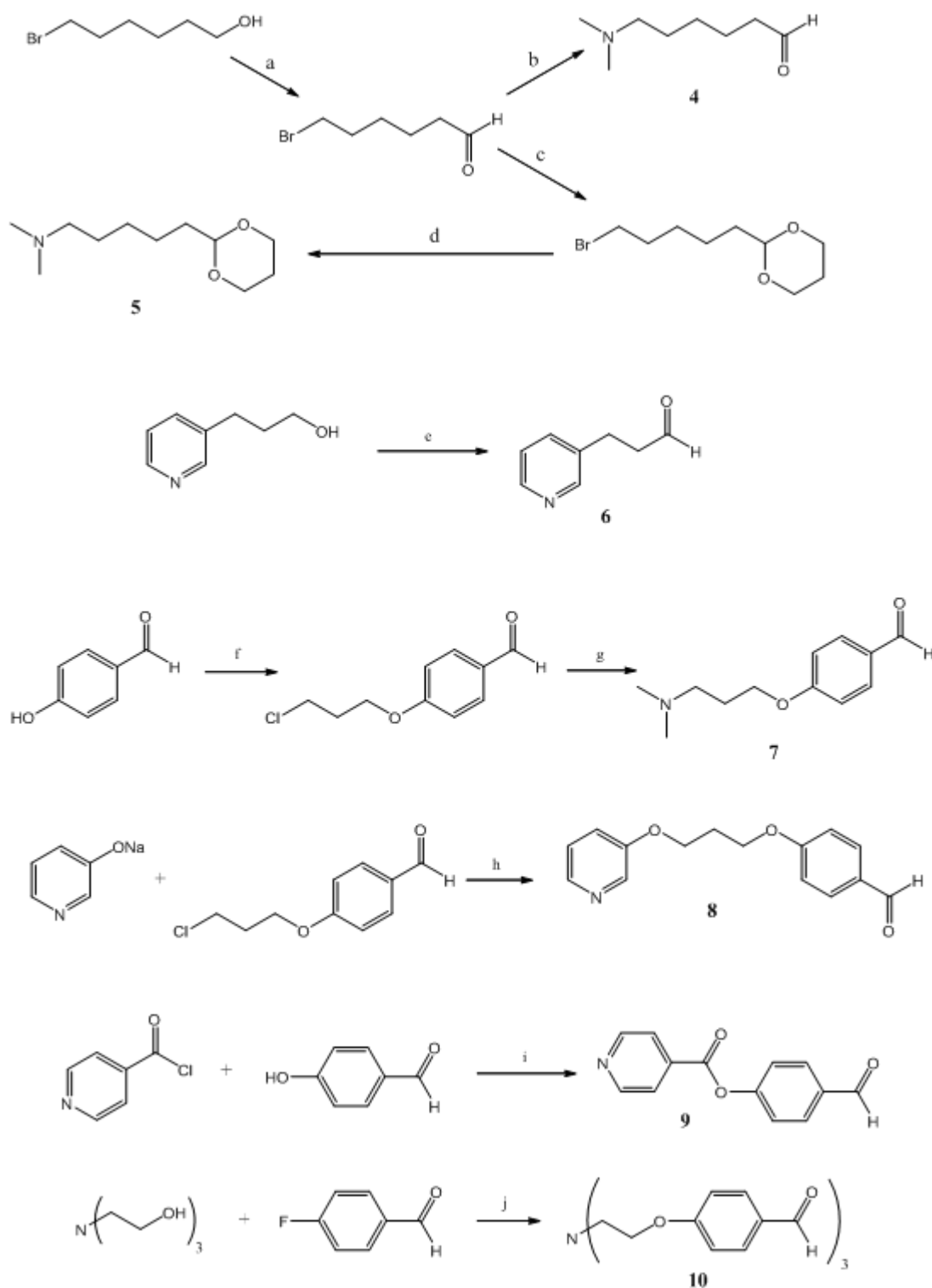
Figure 12. Synthetic schemes for tertiary amines prepared for "click" chemistry synthesis. (a) oxalyl chloride, DMSO, CH_2Cl_2, -78 °C, 1h; (b) $HN(CH_3)_2$, EtOH, reflux 18h; (c) 1,3-propanediol, pyridinium p-toluene sulfonate, toluene, reflux, 18h; (d) $HN(CH_3)_2$, EtOH, reflux 18h; (e) oxalyl chloride, DMSO, CH_2Cl_2, -78 °C, 1h; (f) K_2CO_3, acetone, reflux, 18h; (g) $HN(CH_3)_2$, EtOH, reflux 18h; (h) THF, reflux, 18h; (i) Et_3N, CH_2Cl_2, 25 °C, 18h; (j) KOH, DMF, 70 °C, 48h.
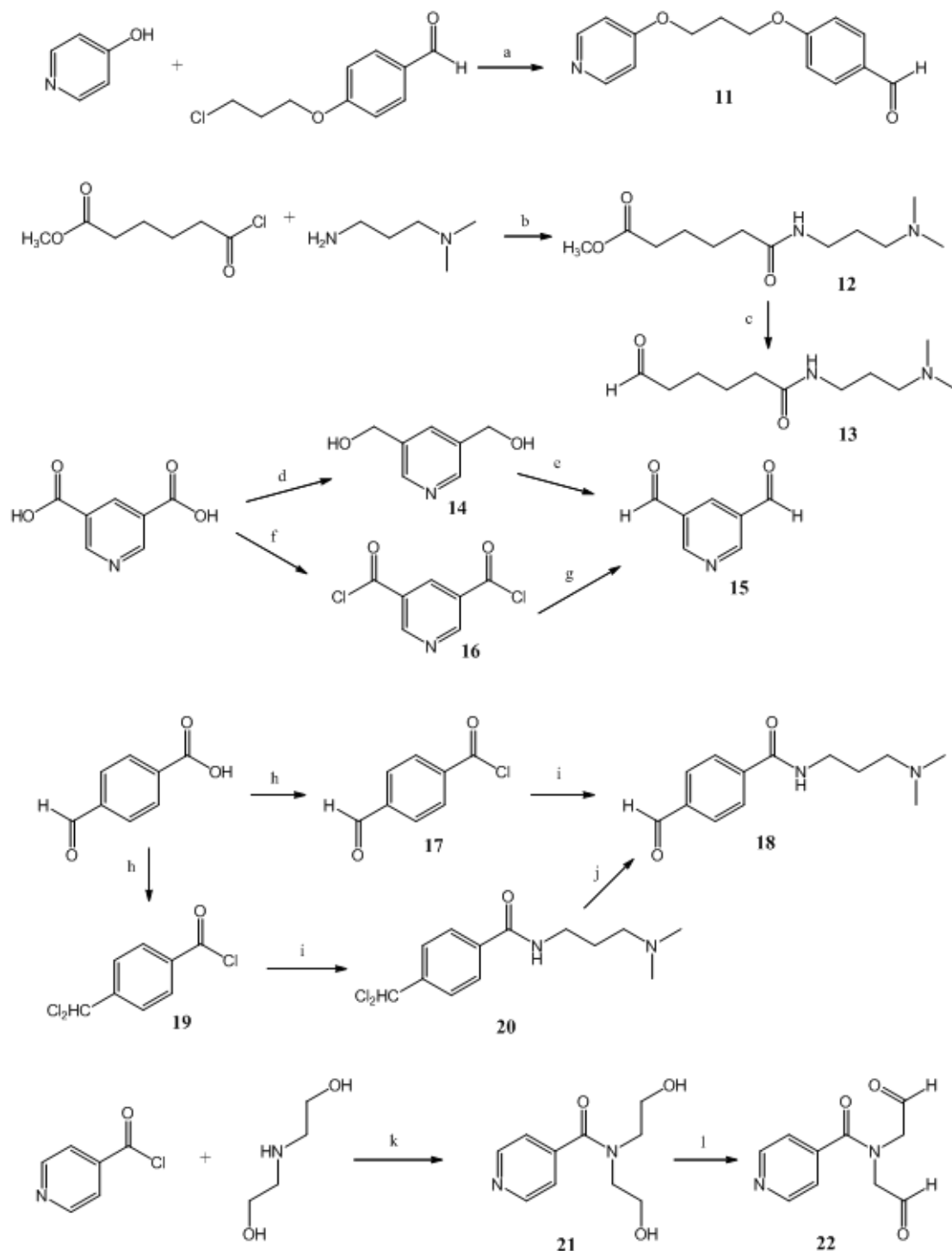
Figure 13. Unsuccessful synthetic schemes for tertiary amines for "click" chemistry synthesis. (a) K_2CO_3, acetone, reflux, 18h; (b) CH_2Cl_2, 25 °C, 18h; (c) di(isobutyl)aluminum hydride, -78 °C, 1h; (d) BH_3, THF, 0 °C, 18h; (e) oxalyl chloride, DMSO, CH_2Cl_2, -78 °C, 1h; (f) $SOCl_2$, reflux 12h; (g) Li tri-tert-butoxyaluminum hydride, THF, -78 °C, 1h; (h) $SOCl_2$, reflux 12h; (i) 3-dimethylaminopropylamine, CH_2Cl_2, 0 °C, 18h; (j) 1M HCl, 25 °C, 12 h; (k) Et_3N, CH_2Cl_2, 25 °C, 18h; (l) oxalyl chloride, DMSO, CH_2Cl_2, -78 °C, 1h.

The various tertiary amine targets shown in Figures 11, 12, and 13 were chosen in order to study the effect of the aldehyde side chain structure on the ability of the coatings to kill spores. These structures contain great variations in both the length and the flexibility of the side chains. In addition, crosslinking of the polymers became an issue (see section 3.2.2) and the amount of steric hindrance around both the aldehyde and tertiary amine were varied in order to avoid unwanted crosslinking.

The reactions shown in Figure 13 are all drawn to show the intended pathways and products. Some discussion is therefore needed to explain why these reactions were considered unsuccessful. Many attempts were made to form ethers from the hydroxyl group on 4-hydroxypyridine. However, 4-hydroxypyridine forms a stable tautomer in which the hydrogen atom from the hydroxyl group is transferred to the nitrogen atom. The stability of this tautomer prevents the oxygen atom from acting as a nucleophile and ethers such as **11**, could not be formed. Synthesis of the ester **12** was successful but attempts to reduce the ester to the aldehyde **13** yielded only starting materials, despite the fact that the formation of aldehydes from esters using DIBAL is a textbook reaction. The dialdehyde **15** was greatly desired since it was similar to compound **1**, but would result in a polymer with twice as many aldehyde groups. The initial attempt to prepare **15** began with the reduction of 3,5-pyridine dicarboxylic acid with borane to give the diol **14**. However, only small amounts of **14** could be isolated (probably due to the high solubility of **14** in water) and these turned out to be insoluble in the solvent (dichloromethane) required for the proposed Swern oxidation to convert **14** into **15**. An alternative route to **15** began with the successful conversion of 3,5-pyridine dicarboxylic acid into the diacid chloride **16**. The reduction of **16** with lithium tri-tert-butoxyaluminum hydride according to the procedure of *Collman et al.* [2004] failed to yield any measurable quantity of the dialdehyde. Preparation of **18** was begun by attempting to convert 4-carboxy benzaldehyde into the acid chloride **17** using thionyl chloride. Upon inspection of the product of this reaction however, it was determined that the actual product was **19**. Since benzylic dichloromethyl groups are reported to be easily hydrolyzed into aldehydes, the amide **20** was prepared as a precursor to the original target, **18**. Unfortunately, conditions to hydrolyze the bezylic dichloromethyl group in **20** without also hydrolyzing the amide could not be found. Finally, the dialdehyde **22** was also greatly desired since the two aldehydes were separated by four relatively flexible bonds, making it a good analog of glutaraldehyde. Unfortunately, the diol **21** could not be isolated cleanly, probably due to the formation of a mixture of esters and amides as well as the high solubility of **21** in water.

3.2.2 Polymer Synthesis by "Click" Chemistry

The aldehyde side chains were attached using the same conditions that were used to attach the alkyl side chains described in section 2.1.1. Br-TMPS was dissolved in DMF and a twofold excess of the tertiary amine was added to the solution. The reaction mixture was stirred at room temperature for 24 hours. After isolating the polymer, it was re-dissolved in methanol or acetonitrile without the use of a surfactant. During this procedure, several of the reaction mixtures gelled, apparently due to a crosslinking

reaction. The exact nature of this reaction is still undetermined but it seems to be due to an interaction between the QA and aldehyde groups. One reason for the variety of structures in Figures 11-13 was to determine the nature of this crosslinking and to see if it could be avoided. It was hypothesized that by adding steric bulk around either the aldehyde (by using a benzaldehyde) or the tertiary amine (by using a pyridine) the interactions between these two species could be reduced and the crosslinking could be avoided. The results appear to validate the hypothesis since compounds **4**, **6**, and **7** all resulted in gels when they were added to a solution of Br-TMPS. Compounds **4** and **6** were the only two that did not have benzaldeyhde groups and **7** had the tertiary amine that was not part of a pyridine group. Compounds **3** and **10** also had non-pyridine amines but they each had much more steric bulk nearby than **7**. In addition to the three compounds that formed gels, compound **2** was also not successfully attached to Br-TMPS. This was due to the electron-withdrawing effect of the aldehyde group in the 4-postion on the aniline which reduced the nucleophilicity of the amine so much that it did not react with Br-TMPS, even at elevated temperatures. The remaining six compounds from Figures 11 and 12 (**1**, **3**, **5**, **8**, **9**, and **10**) were all successfully attached to Br-TMPS and the resulting polymers were named PSAx, where x is the number of the tertiary amine from Figures 11 and 12.

PSA5 was a special case since it contained an aldehyde protected as an acetal. Several attempts were made to hydrolyze the acetal with PSA5 in solution but the products were always found to be insoluble and therefore unusable. This was likely the result of crosslinking since the deprotected form of **5** was identical to **4** which formed a gel when mixed with Br-TMPS. In a final attempt to prepare a testable coating from PSA5, the acetal form of PSA5 was sprayed onto a test surface and the coated coupons were then soaked in 0.5 M HCl to hydrolyze the acetal, although no surface analysis was performed to determine if any aldehydes were present.

3.3   Biological Testing

Testing of the aldehyde coatings against spores was similar to that done with vegetative bacteria in chapter 2. An aqueous suspension of organisms was deposited on a test surface for a given amount of time, after which the test coupon was immersed and vortexed in a wash buffer solution and the number of viable organism in the solution was determined by plating. Initial testing with CARC coated coupons showed a surprising decrease in the number of spores (up to 50 %) on the control surfaces which was probably due to the embedding of spores in the porous CARC surface. Such embedded spores, while still viable, could not be washed off of the surface and therefore would not be counted in the plating procedure. After this discovery, the coupons onto which the coatings were sprayed were changed to glass slides (1.75 in.^{2} surface area) with the control surfaces as uncoated glass. All coatings were sprayed to achieve polymer coverages of 5 mg/in^2.

Data from the spore testing is summarized in Table 2. The percent kill was calculated from the difference between the numbers of living colony forming units (CFUs) on the control and coated coupons after a given exposure time on the surfaces. Some cells in Table 2 contain two entries because those coatings were tested twice.

Table 2. Spore testing data for aldehyde polymer coatings.

Polymer	DF	Kill % (1h)	Kill % (3h)	Kill % (6h)
PSA1	1.24	6	38	53
PSA1	1.64	11	56	57
PSA1	1.94	24 / 5	60 / 33	62 / 47
PSA1	2.15	28 / 12	72 / 29	81 / 32
PSA3	1.24	8 / 0	12 / 18	24 / 11
PSA3	1.64	11 / 7	21 / 3	18 / 23
PSA3	2.15	7	30	42
PSA5[a]	1.48	-12	1	3
PSA5[b]	1.48	-13	-3	7
PSA8	1.24	15	31	20
PSA8	1.64	19	19	16
PSA8	1.94	33	62	47
PSA8	2.63	17 / 8	43 / 33	52 / 48
PSA9	1.24	14 / 14	50 / 30	48 / 34
PSA9	1.94	20	24	46
PSA9	2.63	27 / 6	27 / 11	29 / 16
PSA10	1.48	15	18	22
PSE6	0.6[c]	3	5	8

[a] Samples not treated with HCl.
[b] Samples soaked in 0.5 M HCl for 1h.
[c] Effective DF calculated from the number of hydrolyzed acetals.

The DF values in Table 2 are the same degree of functionalities discussed in section 2.1.1; best defined as the number of sites for QA group attachment per polymer repeat unit. Hence, polymers with higher DF values have higher concentrations of QA groups and, consequently, higher concentrations of aldehydes. It was possible to prepare PSA polymers from samples of Br-TMPS with DF>1.48 (unlike the PS-QA polymers in chapter 2) because the aldehydes and their bulky connecting groups rendered these polymers less soluble in water than their PS-QA analogs.

The coating with the highest kill percentage recorded was PSA1 with a DF of 2.15 (81% after 6h). The PSA1 series showed a clear trend toward high kills as the DF increased. Although this would seem to be a rather obvious result, several of the polymers (the PSA9 series, for example) surprisingly did not have better kills at higher DFs. Given these results with PSA1, synthesis of a batch of PSA1 with DF > 2.15 was attempted but the resulting polymer was very hydrophilic and could not be isolated cleanly.

Another unanticipated result was that using tertiary amines with longer, more flexible linkers than **1** did not increase the kill percentages. PSA8 for example, had the amine and aldehydes on separate rings with a flexible 5-atom spacer between them, but the highest kill percent recorded for any of the PSA8 coatings was only 62 %.

PSA5 showed virtually no sporicidal activity whether it was treated with HCl to hydrolyze the acetal or not. As mentioned before, no analysis was performed to check the effectiveness of the hydrolysis, but it was determined that any, more-vigorous hydrolysis that might be required would add an unwanted step in the preparation of the coatings, so the PSA5 coating was not investigated any further.

It is interesting to note that the PSE6 coating, which did not have any QA groups on it, was ineffective at killing spores. This could have been due to the low DF value for PSE6 (0.6), although this highlights one of the shortcomings of the ether side chain approach. Namely, that the maximum possible DF is one unless each side chain contains more than one aldehyde. The lack of sporicidal activity with PSE6 could also be due to the fact that it has no QA groups and, hence, no positive charge. A positive charge is known to greatly facilitate close contact between QA biocides and the negatively-charged membranes of vegetative bacteria, so this could also be why the QA groups on the PSA coatings increased their sporicidal activities relative to PSE6.

Nearly all of the coatings that were tested a second time showed lower kill percentages on the second test. The time that elapsed between these tests varied from about 4 to 8 weeks so an exact relationship between performance and polymer age was difficult to determine. But the fact that the sporicidal activities decreased so dramatically over such a short time probably indicates that the stability of the aldehyde groups is too low for these coatings to be of practical use. Given that the polymer solutions were all stored in a refrigerator at 10 $^{\circ}$C during the intervals between tests, degradation of the aldehyde groups would occur even faster in most end-use environments.

3.4   Conclusions

The most effective method for preparing poly(sulfone)s with pendant aldehydes was the "click" chemistry route wherein a polymer with benzylic bromomethyl groups was treated with a tertiary amine to form QA groups. The tertiary amines were part of larger molecules which also had aldehydes or aldehyde precursors attached to them. Several of the aldehyde polymers were found to kill >50 % of the spores placed in contact with them although their sporicidal activities decreased significantly over a 4 to 8 week interval. It was also found that the polymers with both QA and aldehyde groups were much more effective at killing spores than were polymers with only aldehyde groups.

3.5   Experimental

3.5.1 Materials

4,4'-Isopropylidenebis(2,6-dimethylphenol) (tetramethyl bisphenol A or TMBPA) was recrystallized from toluene and dried under vacuum. 4-Fluorophenyl sulfone was recrystallized from ethanol and dried under vacuum. 2-Methoxyhydroquinone was purified by sublimation. All other reagents and solvents were purchased from commercial vendors and used without further purification. TMPS and Br-TMPS were prepared as described in section 2.4.1. PSA polymers were prepared and isolated using the same procedure described for the PS-QA polymers in section 2.4.1.

Synthesis of 6-dimethylamino-1-hexanal (**4**)
Oxalyl chloride (9.6 ml, 110 mmol) was mixed with 188 mL dichloromethane and chilled in a dry ice/acetone bath under Ar. DMSO (17.3 g, 221 mmol) was added via syringe and the solution was stirred cold for 2 min. 6-Bromo-1-hexanol (10.0 g, 55.2 mmol) dissolved in 50 mL dichloromethane was added and the reaction was stirred for 40 min. Triethylamine (39 mL) was added and the reaction was allowed to warm to room temperature over 2h. Water (60 mL) was added, the reaction was mixed well, and the organic layer was washed with brine and then with water. It was then dried with $MgSO_4$ and the solvent was removed by rotary evaporation. The resulting residue was dissolved in hexane and washed with brine and then with water. The organic layer was dried over MgSO4 and the solvent was removed by rotary evaporation to yield 6-bromo-1-hexanal.

6-Bromo-1-hexanol (2.7 g, 15 mmol) was dissolved in 125 mL ethanol. Dimethylamine (13.2 mL of a 40% solution in water, 104 mmol) was added and the reaction was heated at reflux overnight. The solvent was removed by rotary evaporation and the residue was mixed with 1g Na_2CO_3 dissolved in 40 mL water. The product was extracted into chloroform and was then dried over $MgSO_4$ prior to solvent removal by rotary evaporation to yield 6-dimethylamino-1-hexanal as a yellow oil.

Synthesis of 6-dimethylamino-1-hexanal propylene acetal (**5**)

6-bromo-1-hexanal (6.47 g, 36.14 mmol)(preparation described above) was combined with 1,3-propanediol (13.75 g, 181 mmol), pyridinium p-toluenesulfonic acid (1.82 g, 7.2 mmol), and toluene (250 mL) in a flask with a Dean-Stark trap. The reaction was heated at reflux for 18 h and then the solvent was removed by rotary evaporation. The resulting residue was mixed with 200 mL ether and washed with saturated $NaHCO_3$ and then with water. The organic layer was dried over MgSO4 and the solvent was removed by rotary evaporation to yield 6-bromo-1-hexanal propylene acetal.

6-Bromo-1-hexanal propylene acetal (5.22 g, 22 mmol) was dissolved in 200 mL ethanol. Dimethylamine (20 mL of a 40% solution in water, 158 mmol) was added and the reaction was heated at reflux overnight. The solvent was removed by rotary evaporation and the residue was mixed with 1g Na_2CO_3 dissolved in 40 mL water. The product was extracted into chloroform and was then dried over $MgSO_4$ prior to solvent removal by rotary evaporation to yield 6-dimethylamino-1-hexanal propylene acetal as a yellow oil.

Synthesis of 3-pyridinepropanal (**6**)

Oxalyl chloride (3.6 ml, 41 mmol) was mixed with 45 mL dichloromethane and chilled in a dry ice/acetone bath under Ar. DMSO (6.8 g, 87 mmol) was added via syringe and the solution was stirred cold for 15 min. 3-Pyridinepropanol (5.0 g, 36 mmol) dissolved in 45 mL dichloromethane was added over 20 min. and the reaction was then stirred for 45 min. Triethylamine (39 mL) was added and the reaction was allowed to warm to room temperature over 2h. The reaction mixture was washed with water (3 x 80 mL), dried over $MgSO_4$ and the solvent was removed by rotary evaporation to yield 3-pyridinepropanal as a dark oil.

Synthesis of 3'-dimethylamino-4-propoxybenzaldehyde (**7**)

3'-chloro-4-propoxybenzaldehyde (2.50 g, 12.6 mmol) (prepared according to the procedure of *Ouach et al*. [2008]) was dissolved in 100 mL ethanol. Dimethylamine (11.1 mL of a 40% solution in water, 88 mmol) was added and the reaction was heated at reflux overnight. The solvent was removed by rotary evaporation to yield the title compound as a yellow oil.

4''-carboxaldehyde-3'-phenoxy-3-propoxypyridine (**8**)

3-Hydroxypyridine sodium salt (5.89 g, 50.3 mmol) and 3'-chloro-4-propoxybenzaldehyde (5.00 g, 25.2 mmol) (prepared according to the procedure of *Ouach et al*. [2008]) were combined with 50 mL DMF and stirred for 1.5h at room temperature and then at 80 $^{\circ}$C for 18 h. The reaction mixture was poured into 200 mL water and was then extracted with ether. The ether was removed by rotary evaporation to yield the title compound as a yellow oil.

4-carboxaldehyde-phenylisonicotinoate (**9**)

Triethylamine (12.5 mL, 89.9 mmol) was added to a solution of isonicotinoyl chloride·HCl (8.00 g, 44.9 mmol) in 80 mL dichloromethane in an ice/water bath. 4-Hydroxybenzaldehyde (5.49 g, 44.9 mmol) mixed with 80 mL dichloromethane was added over 5 min. and the reaction was heated to reflux and stirred for 1 h. After cooling

to room temperature, 1g Na_2CO_3 dissolved in 100 mL water was added to the reaction and mixed. The organic layer was washed with water, dried over $MgSO_4$ and the solvent was removed by rotary evaporation to yield the title compound as a gray solid.

Tris(4'-carboxaldehyde-2-phenoxyethyl)amine (**10**)

Triethanolamine (2.10 g, 14.1 mmol) was combined with 4-fluorobenzaldehyde (7.00 g, 56.4 mmol), potassium hydroxide (3.16 g, 56.4 mmol), and DMF (100 mL). The mixture was heated at 60 $^{\circ}$C under Ar for 18 h. After cooling to room temperature, the mixture was poured into 350 mL water and then extracted with ethyl acetate (3 x 50 mL). The extract was washed with brine, dried over $MgSO_4$ and the solvent was removed by rotary evaporation to yield an orange oil. The residue was mixed with 1 M HCl (100 mL) and 50 mL chloroform. NaOH (1M) was added to the aqueous layer until the pH was greater than 7. The cloudy mixture was then extracted with dichloromethane (2 x 100 mL). The extract was dried over $MgSO_4$ and the solvent was removed by rotary evaporation to yield the title compound.

3.5.2 Characterization

Gel permeation chromatography (GPC) was performed with a liquid chromatograph equipped with a Viscotek VE2001 isocratic pump and autosampler and a Viscotek VE3580 refractive index detector. The mobile phase was tetrahydrofuran and the system was operated at 25 °C with a flow rate of 1.0 mL min^{-1}. The weight-average molecular weights were measured by calibration with polystyrene standards.

^{1}H NMR spectra of the polymers were obtained on a Bruker spectrometer using 5 mm o.d. tubes. Sample concentrations were about 5 % (w/v) in $CDCl_3$ containing 1 % TMS as an internal reference.

3.5.3 Spore Testing Protocols

General test preparation (**best done the day before**)
1. Label petrifilm.
2. Place 30 50-ml tubes on plastic risers with lose caps. Place the conicals on their sides and tape to board to prevent the tubes from rolling.
3. Locate five (5) polymeric coated coupons and five (5) blank CARC coupons in sterile 50-ml screw-top conical tubes for each time frame (1h, 3h, and 5h).
4. Place five empty 50-ml tubes in an upright rack for each time frame.
5. Label tubes.
6. Make 2 or more liters of sterile phosphate buffer solution (PBS).
7. Make 5 or more racks of DI water blanks and autoclave.

Microorganism preparation
1. Sterilize a 250-ml glass jar with lid (or use a sterile plastic jar).
2. Add 450 ml sterile DI water

3. Add 1 ml spore stock from Apex laboratories (e9).
4. Vortex
5. Store in the refrigerator.
6. Plate dilutions to confirm concentration of e7.

Surface inoculation
1. Locate five (5) polymeric coated coupons, five (5) blank (CARC) coupons in sterile 50-ml screw-top conical tubes and five empty 50 ml tubes.
2. Inoculate each coupon with 500 µl of the prepared bacterial suspension ($\sim10^7$ CFM per ml).
3. Place cap loosely on conical.
   Inoculate the 6 hour contact tubes first, then the 4 hour tubes and finally the 1 hour tubes.
4. Start timer once both the polymer coated and control coupons (including the 5 controls in the upright rack) for each time have been inoculated.

Microorganism Extraction and Analysis
At the end of the contact period:
1. Place conical upright in rack
2. Add 22 ml PBS buffer with 0.01% Tween 80.
3. Seal with a cap.
4. Extract immediately by vortexing for 15 seconds.
5. Prepare serial dilutions of the extracted suspension (dilutions 1-4).
6. Plate 1.0 ml of the original suspension and each dilution to Petri film.
7. Incubate plates at 37°C in ambient air and examine after 48 hours.
8. Count colonies on the countable plates (30 to 300 CFU/ plate).
9. Calculate CFU (plate count x dilution factor x 30 = Total CFU per sample).
10. Enter counts into the spreadsheet.

4 SUMMARY/CONCLUSIONS/NEXT STEPS

The work presented in this report shows that:

- Poly(sulfone)s with quaternary ammonium groups can be synthesized and sprayed to form coatings that kill 99.9% of both gram positive and gram negative bacteria on contact.

- These coatings still kill 99.9% of bacteria after 14 days of exposure to a simulated diurnal temperature cycle and they still kill 87% of bacteria after 14 days of immersion in water.

- These same polymers can be modified using a "click" chemistry approach to add aldehyde functional groups.

- The poly(sulfone)s with pendant aldehydes can be sprayed to form coatings that kill up to 81% of B. atrophaeus spores on contact within 6 hours.

- The combination of quaternary ammonium groups and aldehydes is required for the coatings to kill any significant amount of spores.

The aldehyde coatings represent the first report of a sporicidal coating that does not rely on the release of biocides into the environment. The percentage of spores killed is perhaps not high enough to be of practical use and the long-term stability of the coatings has not been thoroughly investigated. Future work on these coatings should include efforts to develop ammonium groups that have two or more aldehydes attached to them in order to increase the ability of the coating to kill spores. Also, trialkoxysilane groups can be attached to the polymers in order to allow the coatings to covalently bond to surfaces with hydroxyl or amine groups (such as cotton and glass). Additionally, the possible use of coatings with aldehydes protected as acetals could be explored. The rate at which the acetals hydrolyze to aldehydes could be tuned to extend the usable lifetime of the coatings.

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