Designing Antimicrobial Polymer Coating to Inhibit Pathogenic and Spoilage Microorganisms

Anne Yu-Ting Hung

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DESIGNING ANTIMICROBIAL POLYMER COATING TO INHIBIT PATHOGENIC AND SPOILAGE MICROORGANISMS

A Thesis Presented

by

ANNE YU-TING HUNG

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

February 2018

Food Science
DESIGNING ANTIMICROBIAL POLYMER COATING TO INHIBIT PATHOGENIC
AND SPOILAGE MICROORGANISMS

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Food Science
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ABSTRACT

DESIGNING ANTIMICROBIAL POLYMER COATING TO INHIBIT PATHOGENIC AND SPOILAGE MICROORGANISMS

FEBRUARY 2018

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Microbial cross-contamination remains an on-going challenge in the food sector despite implemented sanitation programs. Antimicrobial coatings with inherent self-sanitizing properties have been explored to enhance current cleaning practice and support food safety. Prior work has demonstrated successful incorporation of dual antimicrobial characters, cationic polymers and N-halamines, into one coating system. In addition to the rechargeable nature of N-halamines, the coating was reported to exhibit biocidal effects due to the inherently antimicrobial cationic moieties and the chlorinated N-halamines. However, while these polymer coatings were able to retain antimicrobial activity after repeated chlorination, signs of hydrolysis was observed for the N-halamine bonds, indicating potential issues for long-term usage. Herein, we introduced varied molecular weight cross-linkers in an adaption of the established fabrication method to evaluate cross-linker molecular weight (styrene maleic anhydride (SMA) of 6, 8, 120, 250 kDa) influence on surface properties of the coating.

All antimicrobial polymer coatings exhibited similar FTIR spectra, with a prominent absorption band at ~1650 cm⁻¹ suggesting successful cross-link of the polyethyleneimine and SMA. Surface concentration of primary amines ranged from 350-900 nmol/cm², and N-halamines from 90-130 nmol/cm². Surface energy decreased with increasing molecular weight of SMA, but
were not statistically different from one another. In the end, optimal cross-linker molecular weight was determined based on antimicrobial performance, where the coated PPs with 6 kDa SMAs demonstrated enhanced biocidal effects against *E. coli* O157:H7 in its chlorinated form. Further, the antimicrobial coating demonstrated efficacy of ~3 to >5 log reductions of microbial load in its unchlorinated and chlorinated form against *E. coli* O157:H7, *L. monocytogenes*, and *P. fluorescens*. Storage studies support the stability of the chlorinated halamines, with full retention of chlorinated N-halamines over a 24 h study (representative of time between sanitation cycles). These results support the potential application of this antimicrobial polymer coating in food processing and handling operations, in support of reducing cross-contamination of spoilage and pathogenic microorganisms.

Keywords: Antimicrobial coatings, N-halamines, Cationic polymers, Microbial inactivation
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LIST OF ABBREVIATIONS

PP- Polypropylene
PEI- Polyethyleneimine
SMA- Styrene maleic anhydride
MA- Maleic anhydride
EEDQ- 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline
MES- 2-(N-Morpholino)ethanesulfonic acid sodium salt
AO7- Acid orange 7
DPD- N,N-Diethyl-p-phenylenediamine
TSB- Tryptic soy broth
TSA- Tryptic soy agar
ATR-FTIR- Attenuated total reflectance Fourier transform infrared spectroscopy
SEM- Scanning electron microscopy
CHAPTER 1

INTRODUCTION

Reducing microbial cross-contamination of pathogenic and spoilage microorganisms from food contact surfaces remains a significant challenge in the food industry. Microorganisms are capable of colonizing solid surfaces and form stable biofilms, which support their viability and growth\textsuperscript{1,2}, indicating that food contact surfaces in food processing environments such as containers, working benches, conveyor belts etc., are all capable of harboring and potentially transferring microbes into the final food product. Contact surfaces contaminated with microorganisms may lead to serious public health problems, which are estimated to cause 48 million illnesses, and of these, 3000 deaths, annually\textsuperscript{3}. There is a financial cost to food borne illness as well, with a reported $16.3 billion dollars to be associated with illnesses caused by just the major foodborne pathogens alone\textsuperscript{4}. In addition, cross-contamination by food spoilage organisms has a huge financial and environmental impact in terms of economic losses from product recalls and food wasted due to microbial spoilage. In order to reduce the likelihood of microbial cross-contamination from food contact materials, both physical and chemical strategies are employed in cleaning and sanitation\textsuperscript{2}, including application of ultrasound\textsuperscript{5}, irradiation\textsuperscript{6} etc., and use of disinfectants like quaternary ammonium compounds\textsuperscript{7}, hydrogen peroxide, and chlorine-based compounds\textsuperscript{8}. Yet, despite the implementation of well-established cleaning protocols, clean surfaces can be immediately soiled by a contaminated product, causing cross-contamination to remain a major issue in maintaining food safety and quality. Hence, the application of antimicrobial coatings on food contact materials poses as a promising opportunity to minimize microbial contamination and further support current sanitation practices in food processing environments.
Antimicrobial coating materials are a form of active food contact application, which features antimicrobial properties that may reduce, inhibit, or retard microbial growth upon interaction with internal environment or surface contact\textsuperscript{9}. A number of antimicrobial coating materials have been proposed to address contamination issues in the food industry. Among these, incorporation of antimicrobial substances in the coating matrix which exert their biocidal effect by migration of the antimicrobials represents a main strategy that has been explored\textsuperscript{10-12}. Although studies have demonstrated the antimicrobial effectiveness of these type of coatings, reliance on leaching of the antimicrobials indicate eventual loss of antimicrobial activity. In addition, active compounds that may migrate into food may be considered and regulated as indirect food additives (Part 170 Title 21 CFR), which further limits use of migratory antimicrobial materials in actual industrial practice\textsuperscript{9,13}. More recently, N-halamine based polymeric materials have been receiving considerable attention due to their rechargeable antimicrobial character. N-halamines are antimicrobial moieties characterized by nitrogen functional groups covalently bound to halogens (mostly chlorine, due to better stability\textsuperscript{14})\textsuperscript{15}. Chlorination of the nitrogens in amine, amide, and imide structures establish the formation of antimicrobial N-halamine moieties, which are then able to regenerate antimicrobial character upon each exposure to a chlorine source, providing long term activity\textsuperscript{16}. N-halamines have been reported to exert antimicrobial activity towards a wide range of microorganisms including bacteria, fungi, viruses, and prevent bacterial biofilm formation\textsuperscript{17,18}. While their exact inactivation mechanism is not yet confirmed, it is generally agreed that two modes of action are involved, including a direct oxidation of microbial membrane from the bound halogens, and penetration of dissociated halogens which disrupts vital biomolecules within the microorganism\textsuperscript{19,20}. In terms of food-related applications, N-halamine materials represent an opportunity for long-term usage as antimicrobial food contact materials, where their activity can be regenerated via exposure to common chlorine-based sanitizers (e.g. bleach). However, some potential drawbacks,
including lack of antimicrobial property in their unchlorinated form, and their chemical integrity and stability after repeated chlorination, remains a significant challenge\textsuperscript{21}.

Cationic polymers are inherently antimicrobial due to their positive charge, which generates interests because it is able to retain antimicrobial efficacy after immobilizing onto a substrate matrix. Inactivation mechanism of these antimicrobials is attributed to ionic exchange between the positive charges of the cationic polymer and the critical cations within the microbial membrane, resulting in a loss of membrane integrity and cell disruption\textsuperscript{22,23}. Cationic polymers have been reported to demonstrate broad spectrum efficacy, exhibiting biocidal effects against Gram-positive and -negative bacteria, yeast, and fungi\textsuperscript{24,25}. Yet, despite the reported antimicrobial effectiveness, their cationic nature also makes them more susceptible to fouling by anionic compounds in organic matter\textsuperscript{21}. This could further promote bacterial adhesion and establish biofilm formation on the surface of the coating material, which increases the risk of cross-contamination, and represents a major hurdle for application in food contact materials, as food systems often contain a range of anionic compounds.

Previous work has reported successful incorporations of both N-halamine and cationic moieties into the same coating system, which were able to demonstrate the rechargeable characteristic of N-halamines and retain antimicrobial properties in its unchlorinated form via cationic polymer components\textsuperscript{26,27}. The proposed antimicrobial coatings exhibited the capability of retaining biocidal efficacy after 10 chlorination cycles, and showed promising results when challenged in the presence of organic matter. However, as mentioned above, one of the major drawbacks of these antimicrobial technologies lies within the stability of the bond linking the N-halamine moieties to the surface of the coating substrate, which may weaken upon repeated or continuous exposure to halogen sources. It is noted that even though the antimicrobial activity was retained after multiple chlorination to simulate repeated usage, an absorbance reduction at the peak referring to amides and imides of the N-halamine moieties was observed in the Fourier
transform infrared (FTIR) spectrum of the coating surface. This suggests hydrolysis of the polymer coating components, and implies stability and durability issues for applications in the varied conditions of the food manufacturing environment.\textsuperscript{26} Introduction of higher molecular weight cross-linking agents with different chemical compositions into the coating system represents a way to modify surface properties. Styrene maleic anhydride (SMA), a polymeric anhydride composed of styrene and maleic anhydride units can cross-link polyethylenimine (PEI) to fabricate antimicrobial polymer coatings due to the high reactivity of the anhydride units.\textsuperscript{26, 27} Molecular weight of SMAs with varying maleic anhydride content has been observed to influence rheology and phase behaviors in polymer blends.\textsuperscript{28} Thus, implementation of higher molecular weight SMAs with their different chemical composition as cross-linking agents may potentially improve surface properties of the coating system, as high molecular weight molecules imply more active sites for interaction between the component layers. This generated interest in exploring the influence of cross-linker molecular weight in terms of their effect on surface chemistry and antimicrobial efficacy of the coating, and to characterize material efficacy against pathogenic and spoilage organisms.

We hypothesized that cross-linker molecular weight may influence the surface properties and/or antimicrobial character of the resulting antimicrobial polymer coating. Therefore, an adaption of a previously established coating fabrication protocol was followed in this study to determine an optimal combination of cross-linker molecular weight for the proposed antimicrobial polymer coating.\textsuperscript{27} The prepared coating was characterized for its surface properties, and went through an antimicrobial screening assay to evaluate biocidal efficacy. An optimal SMA molecular weight was selected for the coating system based on antimicrobial performance, and was further challenged against pathogenic and spoilage microorganisms including \textit{Escherichia coli} O157:H7, \textit{Listeria monocytogenes}, and \textit{Pseudomonas fluorescens}, in
both log reduction and inactivation kinetics studies, and subjected to an atmospheric storage study to determine practical applicability in the food industry.
CHAPTER 2

INFLUENCE OF CROSS-LINKER MOLECULAR WEIGHT ON SURFACE PROPERTIES AND ANTIMICROBIAL ACTIVITY

2.1. Materials and methods

2.1.1. Materials

Polypropylene (PP) pellets and 6 kDa styrene maleic anhydride (SMA) copolymer were purchased from Scientific Polymer Products (Ontario, NY, USA). Molecular weights of 80, 120, and 250 kDa SMA copolymer were purchased from Polyscope (Netherlands). Branched polyethyleneimine (PEI), 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2-(N-Morpholino)ethanesulfonic acid sodium salt (MES) was purchased from GenScript Inc. (Piscataway, NJ, USA). Orange (II) dye (AO7) and sodium hypochlorite solution were purchased from Acros Organics (Fair Lawn, NJ, USA). N,N-Diethyl-p-phenylenediamine DPD total chlorine reagent powder (DPD) was purchased from Hach Co. (Loveland, CO, USA). Tryptic soy broth (TSB), Tryptic soy agar (TSA), and neutralizing buffer were purchased from Becton, Dickinson and Company (MD, USA).
2.1.2. Fabrication of PEI-SMA-PEI coating on PP coupon

![Diagram](image)

- **Figure 2.1.** Schematic at each fabrication step of cationic and N-halamine polymer coating. MW represents molecular weight of SMA crosslinker (6, 80, 120, 250 kDa)

**2.1.2.1. Preparation of PP films**

PP pellets were cleaned via sonication with first isopropanol, then acetone, followed by deionized water, with two cycles of 10 min cleaning applied for each solution. Cleaned PP pellets were dried and maintained in a dessicator over drierite (anhydrous calcium sulfate), and then pressed into films on a hot press (Carver Inc., NJ, USA) at 180°C with a force load of 9000 pounds. Pressed PP films were cut into 2 x 2 cm squares, and cleaned and dried again following the same cleaning protocol as applied to the PP pellets.
2.1.2.2. Surface activation of PP

The surface of PP films was first activated to facilitate the binding of the coating. Squares of PP (2 × 2 cm) were treated with UV-ozone irradiation for 15 min on one side using a Jelight Co. Model 42 UVO Cleaner (Irvine, CA, USA), which promotes the formation of carboxylic acids through photo-oxidation. The UVO-treated PP coupons were then subjected to a reaction with EEDQ to create surface anhydride groups, further making the PP surface more reactive towards nucleophilic attack by primary amines groups present in PEI polymer. Briefly, a 0.1 mM solution of EEDQ in 50 mM MES buffer (pH 5.5) was prepared with 50 mL of solution for every UVO-treated coupon (final volume (mL) = number of coupons × 50 mL/coupon). This was done by dissolving the appropriate amount of EEDQ first in methanol (1% of final volume), before mixing into the final volume of pH-adjusted MES buffer to make the EEDQ solution. UVO-treated PPs were then immersed and stirred in the EEDQ solution for 2 h followed by rinsing with 1% methanol solution and drying under compressed house air.

2.1.2.3. Spin-coating assembly of polymer coating

The three layer polymer coating was then assembled onto the anhydride functionalized PP coupons by depositing two layers of PEI cross-linked by a layer of SMA using an adaption of previously reported method\textsuperscript{27}. PEI and SMA solutions were prepared with acetone at the concentration of 0.06 g/mL and 0.04 g/mL, respectively, and sonicated until the polymers were fully dissolved. The polymer solutions were then applied by spin-coating alternating layers of PEI and SMA in the order of PEI, SMA, and PEI, which was operated under the conditions of 3000 rpm and 50 psi for 1 min per layer. Coated films were cured at 165°C for 20 min to enable cross-linking between the amines and the anhydride groups. The surface chemistry of the reported polymer coating is illustrated as followed (Figure 2.2.).
2.1.3. Surface characterization of antimicrobial polymer coating

2.1.3.1. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

Surface chemistry of native and modified PP films was analyzed with an IRPrestige 21 spectrometer (Shimadzu Corp., Tokyo, Japan) equipped with a diamond ATR crystal. For each set of prepared films, two spots were measured for their absorbance in each sample film for at least 5 randomly selected films. FTIR analysis was conducted using Happ-Genzel apodization at a resolution of 4 cm\(^{-1}\) and with a total of 32 scans applied for each measurement. The obtained spectra then underwent base-line correction, smoothing, and were analyzed with KnowItAll software (Biorad Laboratories, Philadelphia, PA, USA).

2.1.3.2. Primary amine and N-halamine quantification

The amount of primary amine on the polymer coating surface was determined by the Acid Orange 7 (AO7) colorimetric assay\(^{29}\), in which the \(-\text{SO}_3\) functional group of the dye
complexes with primary amine units in a 1:1 ratio. Squares of 2 × 2 cm modified PP films were cut into 1 × 1 cm sample films, which were then individually immersed in 1mM solution of AO7 dye adjusted to a pH of 3.0 by HCl, and shaken for 3 h. After rinsing in copious pH 3.0 water to remove the unbound dye, the films were shaken in 5 mL of pH 12.0 deionized water (adjusted with NaOH) for 15 min to desorb the bound dye. Absorbances were read at 455 nm, and primary amine content was quantified by comparison to a standard curve prepared with varied concentrations of AO7 in pH 12.0 water. Native PP films conducted in the same manner served as negative controls. Assays were performed with at least two replicates (1 × 1 cm films) from three separately prepared sets of coated films (n=6).

N-halamine content of the polymer coating was quantified by a colorimetric DPD assay to measure surface chlorine capacity. Modified PPs (1 × 1 cm) were individually exposed to 200 ppm of chlorine prepared from sodium hypochlorite solution, of which the chlorine content was confirmed through standardization by iodometric titration\textsuperscript{39}. After 1 h of chlorination, the chlorinated sample films were rinsed with copious water to remove unbound chlorine, and transferred to individual test tubes with 2 mL of deionized water and 50 µL of DPD reagent (prepared by mixing a packet of DPD total chlorine reagent powder with 1 ml of deionized water). The tubes were shaken for 5 min to allow color formation, and the absorbances were immediately read at 512 nm. N-halamine content was determined by comparison to a standard curve prepared with varied solutions of sodium hypochlorite in deionized water. Native PP films exposed to 200 ppm chlorine served as negative controls. Disposable test tubes were used throughout the assay to minimize chlorine demand. The assays were performed with at least two replicates (1 × 1 cm films) from three separately prepared sets of coated films (n=6).

2.1.3.3. Water contact angles and surface energy

Hydrophobicity of the native and modified PPs was evaluated using advancing water contact angles measured with a DSA100 Drop Shape Analyzer (Krüss, Hamburg, Germany). A
drop of 5 µL HPLC grade water was dispensed onto the sample films at a 25 µL/min rate on a 1 × 2 cm film. Measurement data and images were obtained through the Drop Shape Analysis software (Krüss, Hamburg, Germany). For each type of sample, at least two different spots on a 1 × 2 cm film were tested from three separately prepared sets of films (n=6).

Surface energy of the polymer coating was calculated using the Zisman plot method\textsuperscript{31, 32}. Advancing contact angles were obtained in the same protocol as mentioned above, with the following liquids of different surface tension values: water (72.8 mN/m), ethylene glycol (47.7 mN/m), diethylene glycol (44.8 mN/m), and acetone (25.8 mN/m). The measurements and images were obtained through the Drop Shape Analysis software (Krüss, Hamburg, Germany). At least two different spots on a 1 × 2 cm film were tested from three separately prepared sets of films for each type of PP and liquid. Cosine values of the obtained advancing contact angles were then plotted against the surface tension value of the corresponding liquid and fitted to a linear regression curve with GraphPad software (GraphPad Software Inc., La Jolla, CA, USA). Surface energy of the native and modified PP films were determined when the advancing contact angle equals 0, giving a cosine value of 1.

2.1.4. Antimicrobial evaluation

Antimicrobial activity of the modified PPs were evaluated against \textit{Escherichia coli} O157:H7 ATCC 43895 (provided by Dr. Lynne McLandborough, University of Massachusetts Amherst, USA) and \textit{Listeria monocytogenes} Scott A (provided by Dr. Martin Wiedmann, Cornell University, USA) by exposing unchlorinated and chlorinated sample films to aqueous bacterial suspension, with uncoated PP films, bacterial suspension without films and uninoculated media serving as controls. The antimicrobial assay was conducted following previously established lab protocols\textsuperscript{26, 27}. Briefly, a single colony of tested bacteria was inoculated in TSB and incubated for 16 h at 37°C under shaking (125 rpm) overnight to reach stationary phase. A 1% dilution of this bacterial suspension was then prepared with fresh TSB, and incubated at 37°C again until the
bacteria reached mid-exponential phase (~2 h for *E. coli* and ~4 h for *L. monocytogenes*). The resulting broth was diluted 1000 fold with sterile deionized water for a starting inoculum bacterial concentration of ~6 log (CFU/mL). For each type of bacteria and native and coated PP, four 1 × 1 cm films were submerged in 1 mL of aqueous bacterial suspension. The immersed films were then incubated at 32°C with 60 rpm rotation for pre-determined cutoff time points (1 h for *E. coli* and 20 min for *L. monocytogenes*). At the designated time point, bacterial suspensions were taken from each test tube and serially diluted with first neutralizing buffer to quench the chlorine, followed subsequently by 0.9% saline water. Serial dilutions were then plated out in duplicates on TSA plates. The entire volume (1 mL) of the first dilution of each sample was plated out in triplicates (333 µL per TSA plate) as to lower the limit of detection to 1 log (CFU/mL). The TSA plates were incubated at 37°C for 48 h, and the colonies were counted afterwards to enumerate the viable bacteria. Antimicrobial assays were repeated three times on different days with independently prepared sets of films.

### 2.1.5. Statistical analysis

Analysis of variance (ANOVA) and Tukey’s pairwise comparisons were applied with a 95% confidence interval to determine significant differences between molecular weight of cross-linkers and treatments using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA).

### 2.2. Results and discussion

#### 2.2.1. Surface characterization of PEI-SMA\textsubscript{MW}-PEI polymer coatings

ATR-FTIR spectroscopy was used to characterize the surface chemistry of native PP and PP-PEI-SMA\textsubscript{MW}-PEIs (Figure 2.3.). Native PP films demonstrated absorbance peaks at 2980-2820 cm\textsuperscript{-1} and ~1400 cm\textsuperscript{-1}, which are all characteristic of alkane groups from the carbon backbone (C-H bonds) of propene. After applying the polymer coatings onto PP films, PP-PEI-SMA\textsubscript{MW}-PEIs all exhibited similar FTIR spectra. A noticeable wide absorbance band was
detected for each type of polymer coating at around 3600-3000 cm$^{-1}$, which is most likely contributed by vibration of the hydroxyl bond from carboxylic acids (3400-3200 cm$^{-1}$) introduced through the surface activation of PP, and partially by the N-H bond from amines (3500-3300 cm$^{-1}$) and amides (3320-3270 cm$^{-1}$). After the curing process to enable cross-linking between SMA and PEI, a more prominent peak was observed at ~1650 cm$^{-1}$, which can be attributed to the N-H bond from amides (1680-1630 cm$^{-1}$), and the C=O bond from both amide and imide groups (1670-1630 cm$^{-1}$). This suggests successful covalent bond formation between the maleic anhydride from SMA and the primary amines of PEI, confirming that the curing step promotes cross-linking. Indeed, spectra of all coating variants (prepared with different molecular weight SMAs) were similar. It is worth noting, however, that the spectrum of PP-PEI-SMA$_{250}$-PEI demonstrated a more pronounced peak at ~700 cm$^{-1}$. This absorbance band refers to the alkene and aromatic groups of styrene units from SMA, of which, according to the technical data (Table 2.1) provided, showed that the 250 kDa SMAs possess the highest percentage of styrene units in its copolymer blend out of the selected molecular weights, likely attributing to its larger absorbance intensity at 700 cm$^{-1}$. 
Figure 2.3. FTIR spectra of (a) native PP, and PEI-SMA-PEI polymer coatings on PP prepared with (b) 6 kDa SMA, (c) 80 kDa SMA, (d) 120 kDa SMA, (e) 250 kDa SMA crosslinkers.

<table>
<thead>
<tr>
<th>SMA (kDa)</th>
<th>%weight MA</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td>80</td>
<td>26</td>
</tr>
<tr>
<td>120</td>
<td>26</td>
</tr>
<tr>
<td>250</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 2.1. Percentage weight of maleic anhydride for different molecular weight SMA crosslinkers, with 6 kDa SMA purchased from Scientific Polymer Products and the rest from Polyscope.

The PEI-SMA_{MW}-PEI polymer coatings were further characterized using AO7 dye assay to quantify the surface concentration of primary amines (Figure 2.4.). No measurable amount of
primary amine was detected on the surface of native PP, while the PEI-SMA_{MW}-PEI coatings prepared with 6, 80, 120, and 250 kDa SMA cross-linkers each demonstrated 351.8 ± 37.3, 750.1 ± 64.7, 941.9 ± 147.2, and 348.9 ± 53.9 nmol primary amines per cm², respectively. It is interesting to note that although the concentration of applied PEI (contributes the cationic amine groups) is constant for each molecular weight SMA coating, statistical significant difference is observed between the different molecular weight cross-linker coatings, with PEI-SMA_{6}-PEI and PEI-SMA_{250}-PEI showing comparable amounts of primary amine. A correlation was observed with PEI-SMA_{6}-PEI, PEI-SMA_{80}-PEI, and PEI-SMA_{120}-PEI, where primary amines increased with higher molecular weight cross-linker used. Referring back to the chemical composition of SMA (Table 2.1.), 6, 80, and 120 kDa SMA had similar maleic anhydride to styrene molar ratios, while there appeared to be a practical difference between these and that of the 250 kDa SMA, which may explain the observed deviation. Between PEI-SMA_{6}-PEI, PEI-SMA_{80}-PEI, and PEI-SMA_{120}-PEI, the increasing primary amine content may be attributed to the lower ratio of maleic anhydride with higher molecular weight SMA, implying a lower degree of cross-link between the polymer layers. Chlorine capacity of the coatings was determined via DPD dye assays after the PEI-SMA_{MW}-PEI coatings were exposed and chlorinated with 200 ppm chlorine (Figure 2.5.). This concentration was chosen because federal regulations (Part 178 Title 21 CFR) limit the use of higher chlorine concentrations (>200 ppm available chlorine) on sanitizing food contact surfaces and processing equipment. Again, no measurable amount of N-halamine was observed for native PP chlorinated under same conditions, while the PEI-SMA_{MW}-PEI coatings with 6, 80, 120, and 250 kDa SMA cross-linkers exhibited 133.3 ± 14.9, 134.6 ± 14.2, 87.7 ± 9.4, and 115.7 ± 3.9 nmol of N-halamines per cm², respectively. Although statistical differences were observed, there appears to be no practical difference between coating variants, which exhibited an equivalence of 1.5-2.5 ppm of chlorine. These results supported the FTIR analysis which indicates successful introduction of cationic and N-halamine antimicrobial moieties in the polymer coating.
Figure 2.4. Primary amine content of PEI-SMA<sub>MW</sub>-PEI coatings prepared with 6, 80, 120, and 250 kDa SMA cross-linkers. PP-PEI-SMA<sub>MW</sub>-PEI with the same letters are not significantly different (P>0.05). Values represent means of n=6 determinations with error bars indicating standard deviation.
**Figure 2.5.** N-halamine content of PEI-SMAMW-PEI coatings prepared with 6, 80, 120, and 250 kDa SMA cross-linkers. PP-PEI-SMAMW-PEI with the same letters are not significantly different (P>0.05). Values represent means of n=6 determinations with error bars indicating standard deviation.

Hydrophobicity of the polymer coating surfaces was evaluated via measurements of water contact angles (Table 2.2.). As expected, native PP exhibited a hydrophobic surface (θA = 107.8 ± 2.3) due to its low polar hydrocarbon backbone, which provides inherent hydrophobicity. Comparable results of advancing water contact angles have been reported for PP in prior work. An increase in hydrophilicity was observed for the modified PPs prepared with varied molecular weight SMA cross-linkers (PP-PEI-SMAMW-PEIs). This is likely attributed to the surface activation of PP, a necessary step to enhance surface reactivity of polyolefin, which brought in polar carboxylic groups. In addition, amine groups from PEI, which is introduced through the fabrication process, also contributed more polarity to surface interaction with water. Even though a significantly higher hydrophilicity was noted for each group of PP-PEI-SMAMW-PEI compared with native PP, no statistical difference exists between PP-PEI-SMAMW-PEI with different molecular weight cross-linkers, suggesting cross-linker molecular weight does not significantly affect wetting behavior.

Zisman’s plot approach was applied to measure surface energy of the polymer coating. This was calculated via extrapolation from a linear regression model, which was plotted with surface tensions of different liquids and cosine values of their corresponding contact angles. According to this methodology, surface tension of the PEI-SMAMW-PEI coatings is defined as that of a liquid when it is just able to spread completely across the film surface (θ = 0, as in cosθ = 1). The obtained value is referred to as the critical surface tension, which is an empirical approximation of surface energy of the tested material. Liquids (water, ethylene glycol, diethylene glycol, and acetone) of varied surface tensions were selected due to their relatively low
toxicity and viscosity (feasibility for automatic dispensing onto film surface), and because of their well-known surface tension values. Critical surface tension of native PP and PP-PEI-SMA\textsubscript{MW}-PEIs are presented as followed (Table 2.2.), with native PP showing comparable results of low surface energy to reported literature\textsuperscript{26,38}. It was initially speculated that more hydrophobicity will be introduced with higher molecular weight SMAs due to more low polar styrene units, and provide lower surface energy for the polymer coatings. According to our results, critical surface tension values did lower slightly with higher molecular weight cross-linkers, however, there was no significant difference between that of PP-PEI-SMA\textsubscript{MW}-PEIs and native PP. It is worth noting that PP-PEI-SMA\textsubscript{MW}-PEIs still demonstrated critical surface tension values similar to native PP in spite of more high energy groups (amine, hydroxyl, and carboxyl groups) introduced onto the surface, indicating the importance of low polar styrene groups from SMA.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Contact angles ((\theta_A))</th>
<th>Critical surface tension (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native PP</td>
<td>107.8 ± 2.3\textsuperscript{e}</td>
<td>20.7 ± 0.9</td>
</tr>
<tr>
<td>PEI-SMA\textsubscript{60}-PEI</td>
<td>88.8 ± 1.3\textsuperscript{abcd}</td>
<td>23.3 ± 0.2</td>
</tr>
<tr>
<td>PEI-SMA\textsubscript{120}-PEI</td>
<td>82.8 ± 3.7\textsuperscript{a}</td>
<td>21.8 ± 3.7</td>
</tr>
<tr>
<td>PEI-SMA\textsubscript{250}-PEI</td>
<td>83.9 ± 3.6\textsuperscript{ab}</td>
<td>20.7 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>83.9 ± 3.8\textsuperscript{abc}</td>
<td>19.5 ± 1.8</td>
</tr>
</tbody>
</table>

Table 2.2. Advancing water contact angles and surface energy of PEI-SMA\textsubscript{MW}-PEI polymer coatings prepared with 6, 80, 120, and 250 kDa SMA cross-linkers. Samples with the same letters for each column are not significantly different (P>0.05). Values represent means of n=6 and n=3 determinations for contact angles and surface energy studies respectively, with error bars indicating standard deviation.
2.2.2. Antimicrobial evaluation of PEI-SMA\textsubscript{MW}-PEI polymer coatings

Antimicrobial activity of the cationic and N-halamine implemented PP-PEI-SMA\textsubscript{MW}-PEIs was characterized against \textit{L. monocytogenes} and \textit{E. coli O157:H7} to differentiate difference in efficacy. Unchlorinated and chlorinated PP-PEI-SMA\textsubscript{MW}-PEIs were challenged against a starting inoculum of \( \sim 6 \) log (CFU/ml) for each bacteria type, with native PP and plain bacterial suspension groups as negative controls. The antimicrobial assay was conducted with mid-exponential phase bacteria to ensure that the observed inactivation is attributed to the antimicrobial coatings and not affected by descent in cell growth. Cutoff time points were determined via a preliminary inactivation kinetic test with PP-PEI-SMA\textsubscript{6k}-PEIs in order to differentiate microbial efficacy of the PEI-SMA\textsubscript{MW}-PEI polymer coatings.

As expected, no antimicrobial effect was observed for bacterial suspensions in contact with native PP for both \textit{L. monocytogenes} and \textit{E. coli O157:H7}. After 20 min of contact, the microbial load of aqueous \textit{L. monocytogenes} suspension was reduced by both unchlorinated and chlorinated PP-PEI-SMA\textsubscript{MW}-PEIs, with the chlorinated providing a \( \sim 3 \) log reduction, and unchlorinated a \( >5 \) log reduction (Figure 2.5.). This result suggested that both cationic and N-halamine moieties were responsible for imparting antimicrobial activities, with the cationic moieties showing dominant effectiveness at the contact time applied. Some variation was observed for chlorinated PP-PEI-SMA\textsubscript{120k}-PEIs and PP-PEI-SMA\textsubscript{250k}-PEIs, which exhibited inactivation between 3 to 5 log (CFU/mL) reductions. This can be explained by the fact that because the 80, 120, 250 kDa SMAs were composed of varying degrees of MA (compared to the 1:1 ratio of MA and styrene units of 6 kDa SMAs), they are not considered alternating copolymers with alternating styrene and MA units. Hence the N-halamine moieties might not be distributed uniformly across the film surface, resulting in varying chlorine content and degree of inactivation.
Evaluation against *E. coli* O157:H7 had the unchlorinated PP PP-PEI-SMA\textsubscript{MW}-PEIs yield 2-3 log reductions in microbial load (Figure 2.6.). Difference in coatings was observed with the chlorinated PP-PEI-SMA\textsubscript{MW}-PEIs, where PP-PEI-SMA\textsubscript{6}-PEI reduced microbial load below limit of detection (>4 log) at 1 h, while the rest presented ~ 2 logs of inactivation. This result indicated that in agreement with assays challenged against *L. monocytogenes*, both cationic and N-halamine moieties were able to impart antimicrobial activities as presented by inactivation with both unchlorinated and chlorinated coating forms. However, biocidal efficacy of both characteristics produced similar inactivation effects for the PP-PEI-SMA\textsubscript{MW}-PEIs with 80, 120, and 250 kDa SMAs, with the exception of the PP-PEI-SMA\textsubscript{6}-PEIs, of which chlorination of the N-halamine moieties was able to demonstrate pronounced antimicrobial effects towards *E. coli* O157:H7 at the designated incubation time. To better understand the mechanism of how the PEI-SMA\textsubscript{6}-PEI antimicrobial polymer coating functions against different microbes, inactivation kinetic studies were conducted in the following chapter to evaluate effects of the coated film and its chlorinated form.
Figure 2.6. Antimicrobial activity of unchlorinated and chlorinated (Cl) PEI-SMA\textsubscript{MW}-PEI coating prepared with 6, 80, 120, 250 kDa SMA cross-linkers against \textit{L. monocytogenes} for 20 min contact time.

![Graph showing antimicrobial activity](image)

Figure 2.7. Antimicrobial activity of unchlorinated and chlorinated (Cl) PEI-SMA\textsubscript{MW}-PEI coating prepared with 6, 80, 120, 250 kDa SMA cross-linkers against \textit{E. coli} O157:H7 for 1 h contact time.

2.3. Conclusion

PEI-SMA\textsubscript{MW}-PEIs antimicrobial polymer coatings with 6, 80, 120, and 250 kDa SMA cross-linkers were fabricated from an adaption of coat-cure method in previous work\textsuperscript{27,26}. The PP-PEI-SMA\textsubscript{MW}-PEIs were analyzed with FTIR to confirm successful implementation of cationic and N-halamine moieties, and their primary amine and N-halamine contents were characterized via colorimetric dye assays. Surface concentration of primary amine groups ranged from \(~350\) nmol/cm\textsuperscript{2} for PP-PEI-SMA\textsubscript{MW}-PEIs with 6 and 250 kDa SMA, to an approximate 900 nmol/cm\textsuperscript{2} for PP-PEI-SMA\textsubscript{120}-PEIs. N-halamine content ranged from \(~90\) nmol/cm\textsuperscript{2} for PP-PEI-SMA\textsubscript{120}-
PEIs (despite having highest amount of primary amines), to around 130 nmol/cm² for that of PP-PEI-SMA\textsubscript{MW}-PEIs with 6 and 80 kDa SMA, which is equivalent to approximately 1.5-2.5 ppm of chlorine. All PP-PEI-SMA\textsubscript{MW}-PEIs demonstrated low surface energy comparable to that of native PP, but showed no statistical differences between different molecular weight cross-linker groups. In the end, optimal molecular weight SMA was selected in terms of antimicrobial performance, where PP-PEI-SMA\textsubscript{6}-PEIs exhibited an enhanced biocidal effect against \textit{E. coli} O157:H7 in its chlorinated form, and was used as the model in subsequent inactivation and storage studies to characterize antimicrobial efficacy against spoilage and pathogenic microorganisms relevant in food safety and quality.
CHAPTER 3

PRACTICAL APPLICATIONS: BIOCIDAL EFFICACY AGAINST PATHOGENIC AND SPOILAGE MICROORGANISMS AND STABILITY OF HALOGENATED HALAMINES UNDER ATMOSPHERIC STORAGE

3.1. Materials and methods

3.1.1. Materials

PEI-SMA-PEI coated PP films were prepared with 6 kDa SMA (PP-PEI-SMAe-PEIs) following protocol described in Chapter 2. Sodium hypochlorite solution was purchased from Acros Organics (Fair Lawn, NJ, USA). N,N-Diethyl-p-phenylenediamine DPD total chlorine reagent powder (DPD) was purchased from Hach Co. (Loveland, CO, USA). Tryptic soy broth (TSB), Tryptic soy agar (TSA), and neutralizing buffer were purchased from Becton, Dickinson and Company (MD, USA).

3.1.2. Inactivation kinetics study

The inactivation kinetics demonstrated by the antimicrobial polymer coating was further evaluated against Escherichia coli O157:H7 ATCC 43895, Listeria monocytogenes Scott A, and Psuedomonas fluorescens FSL W5-0203 (provided by Dr. Martin Wiedmann, Cornell University, USA). E. coli and L. monocytogenes were cultured via the protocol described in Chapter 2. P. fluorescens was cultured following the same method at an incubation temperature of 28°C and 300 rpm shaking to reach mid-exponential phase (~6 h) after the initial 1% dilution with TSB. The mid-exponential broth was diluted 1000 fold with sterile deionized water for a ~6 log (CFU/mL) bacterial suspension, which was then applied to multiple test tubes of unchlorinated and chlorinated sample films (1ml of bacterial suspension to four 1 × 1 cm films of either treatment group). Uncoated PP films and bacterial suspension without films served as controls. Inactivation was carried out at 32°C under a rotation of 60 rpm for 2 h. Test tubes were taken out at various time points, and serial dilutions of the bacterial suspension was prepared with first
neutralizing buffer, then 0.9% saline water as mentioned before and plated in duplicates onto 
TSA plates. The TSA plates were incubated at the corresponding growth temperature (37°C for E. 
coli and L. monocytogenes and 28°C for P. fluorescens) for 48 h, and the number of survivors 
were determined via plate count. Inactivation kinetic assays were conducted three times on 
different days with independently prepared sets of films, and a representative of the three kinetic 
curves is presented in the data results.

3.1.3. Agar overlay assay

An agar overlay assay was performed after antimicrobial evaluation to determine the 
presence of viable bacteria attached on the coating surface. In brief, the modified PP films were 
collected after assessing their antimicrobial activity, and rinsed first with neutralizing buffer and 
then three times with sterile deionized water to clean and remove any unbound bacteria. The 
cleaned films were then placed individually into sterile petri dishes, and TSA was poured onto the 
sample films and incubated at the corresponding growth temperature for each microorganism 
(37°C for E. coli and L. monocytogenes and 28°C for P. fluorescens). At least two sample films 
(from both unchlorinated and chlorinated treatments) challenged against the selected microbes 
were tested for each antimicrobial assay performed. After 24-48 h of growth, the TSA plates with 
sample films were taken out and observed for presence of bacteria.

3.1.4. Scanning electron microscopy (SEM)

SEM was used to evaluate bacterial attachment on the coating surface following 
antimicrobial activity assays. Modified PP films, both unchlorinated and chlorinated, were rinsed 
three times with sterile deionized water to remove unbound bacteria after antimicrobial 
evaluation. Absolute ethanol was then applied to the cleaned samples films to fix any bound 
bacteria on the surface and left to dry. The films were sputter-coated with gold using a 
Cressington Sputter Coater 108auto (Ted Pella, Inc., Redding, CA, USA) under argon for 30 s. 
Samples films were examined with a scanning electron microscope JCM-6000 NeoScope (JEOL,
Japan), and at least 9 images were captured at locations distributed across a 3 × 3 grid pattern on each of triplicate samples.

3.1.5. Stability of chlorinated halamines under storage

Stability of the N-halamine moieties were evaluated through a 24 h storage study. Films of 1 × 1 cm modified PP were individually chlorinated via the protocol as described in the N-halamine quantification section with 200 ppm of chlorine. The chlorinated films were then dried with an air gun, and stored at 4°C and room temperature (~22°C) respectively. At different time intervals, the sample films were taken out, and their chlorine capacity was determined with the DPD assay as described above. For this storage study, at least two sample films from three separately prepared sets of films were tested for each temperature condition (n=6).

3.1.6. Statistical analysis

Analysis of variance (ANOVA) and Tukey’s pairwise comparisons were applied with a 95% confidence interval to determine significant differences between treatments using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA).

3.2. Results and discussion

3.2.1. Evaluation of inactivation kinetics

Inactivation kinetic studies were conducted with PP-PEI-SMA6-PEIs (determined to be the optimal molecular weight combination) against selected pathogenic and spoilage microbes, E. coli O157:H7, L. monocytogenes, and P. fluorescens, to analyze antimicrobial behavior and mechanism. P. fluorescens is an obligate aerobe, and was cultured at 28°C under intensive shaking (300 rpm) for aeration purposes, while E.coli O157:H7 and L. monocytogenes were cultured as described in Chapter 2. Starting bacterial inoculum was prepared with cells from mid-exponential phase to ensure that reduction of microbial load was induced by antimicrobial characteristics of the polymer coating and not by decrease in cell growth. A concentration of 200 ppm chlorine, which is the upper limit of available chlorine allowed for sanitization of food
contact surfaces as regulated by Food and Drug Administration (Part 178 Title 21 CFR), was used to chlorinate N-halamine moieties of the polymer coating to simulate industrial cleaning practice.

As shown in Figure 3.1, chlorinated PP-PEI-SMA₆-PEIs were able to inactivate initial microbial load (~6 log CFU/mL) of *E. coli* O157:H7 below limit of detection, providing a >5 log reduction (>99.999%) after 45 min of contact. Even an approximate 3 log reduction (~99.9%) was observed for the coated PPs after 30-45 min in its unchlorinated state. Biocidal functions of N-halamines and cationic polymers both works by targeting the membrane of microorganisms. Literature has reported N-halamines to exhibit two modes of inactivation, including a direct oxidation of vital components through microbial cell membrane upon contact with the intact N-halamines, or via penetration from the dissociated free chlorine which disrupts cell functionality. On the other hand, cationic polymers exert antimicrobial activity via an ionic exchange between its positive charges and that of the cell membrane surface, causing destabilization.

According to our results, inactivation kinetics of the unchlorinated and chlorinated PP-PEI-SMA₆-PEIs showed similar behavior, demonstrating an initial fast decrease in microbial population which then levels out to a plateau. For the unchlorinated PP-PEI-SMA₆-PEIs, this is in accordance with kinetics observed by other studies, which also indicated biocidal properties imparted by the cationic moieties of the polymer coating. Chlorinated PP-PEI-SMA₆-PEIs required a longer contact time (45 min) to reach a plateau during its inactivation as compared to its unchlorinated state (30-45 min). One possible explanation might be because the dissociation of free chlorine and subsequent penetration of cells occurred at a slower rate in contrast to unchlorinated films, which draws bacteria to the surface faster due to opposite charges. This implied that in addition to its enhanced biocidal effects (> 5 log reduction), chlorinated coatings may also demonstrate enhanced stability in terms of sustained activity and release.

Although cationic polymers owe their antimicrobial character to their charged nature, this charge may also promote fouling by anionic compounds and subsequent establishment of
microbial biofilms. Prior work reported a chemical heterogeneity from this coating fabrication, which neutralizes the expected positive charges, and hence, reduce fouling possibilities.

Unchlorinated and chlorinated PP-PEI-SMA6-PEIs were analyzed with SEM after kinetic studies to evaluate presence of bacterial attachment, coupled with agar overlay assays to determine viability of attached cells. The captured SEM images (Figure 3.2.) showed no evidence of bacterial adhesion, as with the agar overlay in which no bacterial growth was observed.

![Figure 3.1](image)

**Figure 3.1.** Inactivation kinetics of *E. coli O157:H7* after exposure to PP-PEI-SMA6-PEI. Values represent duplicates and data are representative of experiments independently replicated on at least three days.
Both unchlorinated and chlorinated PP-PEI-SMA₆-PEIs were able to achieve a >5 log reduction (>99.999%) in *L. monocytogenes* within the observed 2 h time frame (Figure 3.3.). However, in contrast to the results observed for *E. coli* O157:H7 inactivation, where the chlorinated coatings demonstrated enhanced biocidal activity over its unchlorinated form, unchlorinated PP-PEI-SMA₆-PEIs was able to inactivate *L. monocytogenes* below limit of detection in 15 min, while chlorinated PP-PEI-SMA₆-PEIs took 30 min to obtain the same reduction level of >5 log reduction. It was initially expected that the chlorinated coating, with both cationic and N-halamine characters, will provide a more pronounced antimicrobial effect than its unchlorinated counterpart as was observed with *E.coli* O157:H7. Yet susceptibility to antimicrobials is highly dependent on the nature of each type of microorganism\(^\text{46}\). The observed results may be attributed to the difference in surface charge upon chlorination of the coatings. N-halamines are capable of forming on primary and secondary amines, amides, and imides, with amines reported to form most stable N-halamine complexes\(^\text{15}\). Thus, with primary amines as active sites for both chlorination and protonation, availability of protonated primary amines may decrease upon N-halamine formation, causing a reduction in net positive charge. This may affect the rate that pulls the anionic bacterial membrane to the polymer coating surface for inactivation\(^\text{22}\). In addition, Gram-positive bacteria are generally more susceptible to antimicrobials due to lack of an outer membrane structure, which limits penetration of antibacterial substances into the cell \(^\text{47}\). As a result, unchlorinated coatings were more effective against *L. monocytogenes* than their chlorinated counterparts. SEM images (Figure 3.4.) acquired after the antimicrobial evaluation presented no evidence of bacterial adhesion for the chlorinated coatings. A few cells of *L. monocytogenes*, were observed for the unchlorinated coated PPs (~12 organisms observed over 9 images acquired on 3 independently prepared samples, fewer than 3
organisms per observed field of view). To confirm that the observed adhered cells were not viable, an agar overlay test was conducted, in which agar were laid atop films and incubated for 48 h at 37°C. No growth was observed, confirming that no viable organisms were present on PP-PEI-SMA₆-PEI for both unchlorinated and chlorinated forms.

Figure 3.3. Inactivation kinetic against *L. monocytogenes* after exposure to PP-PEI-SMA₆-PEI. Values represent duplicates and data are representative of experiments independently replicated on at least three days.

Figure 3.4. Scanning electron micrographs of (a) unchlorinated and (b) chlorinated PP-PEI-SMA₆-PEI in contact with *L. monocytogenes*
Inactivation kinetic against *P. fluorescens* was presented (Figure 3.5). Both unchlorinated and chlorinated PEI-SMA₆-PEI coatings exhibited similar kinetic behavior, inactivating the initial microbial load to around 3 log cycles (99.9%) after a gradual reduction over 2 h. As mentioned earlier, responses against antimicrobial agents may alter greatly towards different microorganisms. The tested *P. fluorescens* strain was isolated from cheese curds and environmental sites in a commercial cheese manufacturing plant, which may explain their resistance towards chlorine as sodium hypochlorite is a common food sanitizer in industrial practice. SEM images was acquired after the antimicrobial evaluations, and likewise with *L. monocytogenes*, no evidence of bacterial adhesion was observed for the chlorinated coatings, while a few cells of *P. fluorescens* was captured for the unchlorinated PP-PEI-SMA₆-PEIs (~5 organisms observed over 9 images acquired on 3 independently prepared samples, fewer than 3 organisms per observed field of view) (Figure 3.6). Agar overlay test indicated no growth of bacteria, implying that the attached bacteria observed on the unchlorinated PP-PEI-SMA₆-PEI coatings were also non-viable.
**Figure 3.5.** Inactivation kinetic against *P. fluorescens* after exposure to PP-PEI-SMA$_6$-PEI. Values represent duplicates and data are representative of experiments independently replicated on at least three days

**Figure 3.6.** Scanning electron micrographs of (a) unchlorinated and (b) chlorinated PP-PEI-SMA$_6$-PEI in contact with *P fluorescens*

### 3.2.2. Stability of chlorinated halamines under atmospheric storage

In addition to effective antimicrobial functionality, stability and consistency of its activity is equally important in terms of practical applications in the food manufacturing environment.

The U.S. Food and Drug Administration established several guidelines regarding the cleaning frequency of equipment food contact surfaces and utensils. According to Food Code 2013, food contact surfaces should be cleaned between usage of different types of raw animal food, from raw food to ready-to-eat food, raw fruits and vegetables with time/temperature control for safety food (formerly “potentially hazardous food”), and any time when contamination is suspected. Aside from the conditions stated above, equipment food contact surfaces should be cleaned at least every 4 hours if time/temperature control for safety food is used, with less cleaning frequencies allowed for lower processing temperatures (Table 3.1). Based on these recommendations, chlorinated polymer coatings were tested for its chlorine capacity at different time points under 4°C and room temperature (22°C) to evaluate if its antimicrobial N-halamine moieties could
sustain until the recommended cleaning time (recharged with sanitizers). The results demonstrated no significant difference between each time point and temperature condition tested under atmospheric storage (Figure 3.7.), indicating the stability of the chlorine bond in PP-PEI-SMAe-PEI coating and sustained antimicrobial activity without reduction over storage.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Cleaning Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0°C (41°F) or less</td>
<td>24 hours</td>
</tr>
<tr>
<td>&gt;5.0°C - 7.2°C (&gt;41°F - 45°F)</td>
<td>20 hours</td>
</tr>
<tr>
<td>&gt;7.2°C - 10.0°C (&gt;45°F - 50°F)</td>
<td>16 hours</td>
</tr>
<tr>
<td>&gt;10.0°C - 12.8°C (&gt;50°F - 55°F)</td>
<td>10 hours</td>
</tr>
</tbody>
</table>

**Table 3.1.** Cleaning frequency of the utensils and equipment at the corresponding temperature.

Retrieved from Food Code 2013, U.S. Food and drug administration

![Figure 3.7](image)

**Figure 3.7.** Storage stability of chlorinated PP-PEI-SMAe-PEI films. N-halamine content at varied temperature conditions under atmospheric storage. Values represent means of n=6 determinations with error bars indicating standard deviation.
3.3. Conclusion

A more intensive inactivation kinetics assay was conducted using PP-PEI-SMA₆-PEIs after the initial screening of antimicrobial activity in Chapter 2. PEI-SMA₆-PEI coatings were challenged against select pathogenic and spoilage microbes, and was able to demonstrate a substantial reduction in microbial load for the tested bacteria. Antimicrobial performances of the modified films vary in their unchlorinated and chlorinated state for each type of bacteria, indicating that the nature of microorganism plays an important factor in biocidal efficacy. Although traces of bacteria were observed for the unchlorinated PP-PEI-SMA₆-PEIs which came in contact with L. monocytogenes and P. fluorescens, a further agar overlay confirmed the nonexistence of viable cells on the coating surface, supporting our conclusion that the observed antimicrobial effects were contributed by both unchlorinated and chlorinated forms of the polymer coating. Storage study of the chlorinated PP-PEI-SMA₆-PEIs under atmospheric conditions demonstrated stability of the chlorinated N-halamine moieties in between simulated cleaning frequency. Overall, this cationic and N-halamine incorporated polymer coating with the determined optimal molecular weight SMA cross-linker exhibited solid antimicrobial effectiveness and potential of retaining its antimicrobial activity under recommended sanitizing cycles.
CHAPTER 4

OVERALL CONCLUSIONS

Microbial cross-contamination of pathogenic and spoilage microorganisms in food manufacturing may inflect serious public health problems and financial impact. Food contact surfaces are favorable for bacterial attachment and if occurred, can subsequently contaminate food products. Although current sanitation practice involves multiple technologies to ensure thorough cleaning, clean surfaces can be immediately soiled by a contaminated product, causing cross-contamination to remain a constant challenge in the food sector. Antimicrobial coatings with inherent self-sanitizing functions thus poses as a promising opportunity to address this issue, where it is speculated to enhance current sanitation and cleaning protocols by providing antimicrobial activity.

The proposed antimicrobial polymer coating is fabricated from adaption of a previously reported method, which integrated N-halamine and cationic moieties, both a unique antimicrobial substance, into one coating system to take advantage of both characteristic sides. PEI, featuring functional nitrogen groups for both cationic and N-halamine formation, was cross-linked with SMA, a polymeric anhydride with high reactivity towards amines, and assembled on to a base of PP. Higher molecular weight SMAs were introduced, anticipating enhanced surface properties for the coating system in terms of surface chemistry and antimicrobial activity. The influence of cross-linker molecular weight was characterized for their surface properties and antimicrobial performances. FTIR analysis showed similar spectra shape for all coatings indicating similar surface chemistry. Surface energy values lowered with increasing SMA molecular weight (higher amount of styrene units in composition), however, there was no significant difference between different molecular weight coatings. Antimicrobial activity was then evaluated via screening with cutoff contact times against L. monocytogenes and E.coli O157:H7. In the end, the polymer
coating prepared with 6 kDa SMA was selected based off its enhanced antimicrobial effects against *E.coli* O157:H7.

Further work was completed using the determined optimal cross-linker molecular weight coating (PP-PEI-SMA-PEI) to evaluate practical applicability. The antimicrobial coating was challenged against a selection of pathogenic and spoilage microorganisms to decipher its inactivation kinetics. Biocidal effects varied when tested against different microbes, with the chlorinated coating more effective against *E. coli* O157:H7 (>5 log reduction as compared to 3 logs), unchlorinated more predominant against *L. monocytogenes* (same level of reduction in shorter contact time), and both unchlorinated and chlorinated forms exhibited similar antimicrobial behavior against *P. fluorescens* (~3 log reduction). This indicated that antimicrobial efficacy of the polymer coating is heavily dependent on the nature of each microorganism, and more specific target microbes should be tested, depending on the application of the antimicrobial coating, to better understand its potential in an actual food processing environment. Stability of the chlorinated N-halamines were demonstrated under atmospheric conditions, and were shown to be capable of maintaining the chlorine content in between recommended cleaning cycles.

Overall, the proposed antimicrobial polymer coating demonstrated substantial antimicrobial effects against the tested pathogenic and spoilage microbes, reducing the initial microbial load from a ~3 log reduction (99.9%) to below limit of detection (>99.999%) with its unchlorinated and chlorinated form. In addition to its rechargeable antimicrobial nature, its low surface energy and stability of chlorinated N-halamine makes it a promising material to supplement and enhance current sanitation programs in the food manufacturing environment, further supporting food safety and quality.
CHAPTER 5

FUTURE WORK

As mentioned in Chapter 3, cells of attached bacteria, although non-viable as determined by the agar overlay assay, was observed on the coating surface of unchlorinated PP-PEI-SMA₆-PEI after evaluation against *L. monocytogenes* and *P. fluorescens*. Initial bacterial adhesion can stimulate exopolysaccharide synthesis by bacteria, and further establish biofilm formation, causing cleaning and contamination problems in the industry⁵¹. Thus, the next step in this work would be to understand the extent of fouling that has occurred on the antimicrobial polymer coating, and study its ability to resist biofilm formation. The amount of attached of bacteria detected through SEM images can be roughly quantified to that of an entire 1 × 1 cm coating surface, and compared with a positive control as to evaluate the extent of fouling (or antifouling) demonstrated by the polymer coating. Results from this evaluation will be heavily dependent on the representativeness of the acquired SEM images. Although our images were captured at multiple locations uniformly distributed across a 3 × 3 grid pattern to better represent the entire coating surface, in reality, bacteria adhesion occurs randomly on the surface, and it is nearly impossible to capture every inch of the coating surface. Therefore, the data obtained from this should be treated with reservation. In addition, biofilm formation assays should be conducted to provide a more comprehensive understanding of the antifouling characters of this antimicrobial polymer coating. These will help uncover the potential limitations of the coating, and better assess its applicability as active food contact materials.

The antimicrobial effectiveness of the coating was tested against select pathogenic and spoilage microorganisms in this project, with the polymer coatings exhibiting different inactivation kinetics against different microbes in both their unchlorinated and chlorinated state. As biocidal efficacy varies with each type of bacteria, it is important to evaluate it antimicrobial properties against a wide range of microorganisms, and especially on some target microbes in the
food processing environment since the antimicrobial effects seem to be microorganism-dependent. The antimicrobial coatings should be evaluated for both its unchlorinated and chlorinated form to determine kinetics of inactivation, which will provide insight as to what kind of setting and target microbes it should be applied to in the food industry.

Stability of the chlorinated N-halamines should also be studied in different environmental conditions as to simulate the extreme and varying conditions of the food processing plant. In this work, stability of the chlorinated N-halamines were studied under atmospheric storage at recommended sanitizing time intervals to evaluate if the coating can provide continuous antimicrobial activity until the next sanitation (recharge) cycle. Different storage conditions, such as aqueous, extreme levels of pH (to imitate conditions caused by acidic and alkaline sanitizers) should all be explored. Extensive studies are required in order to evaluate the chemical integrity and preserve antimicrobial activity of the polymer coating.
REFERENCES


