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Spray Fabrication of Layer-by-Layer Antimicrobial N-Halamine Coatings

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Spray Fabrication of Layer-by-Layer Antimicrobial N-Halamine Coatings

A Thesis Presented

By

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DEDICATION

To my parents, thank you for always encouraging me to explore the world.

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ABSTRACT

SPRAY FABRICATION OF LAYER-BY-LAYER ANTIMICROBIAL N-HALAMINE COATINGS

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Antimicrobial coatings in which the active agent (e.g. N-halamine) can regenerate activity represent a promising way to prevent microbial cross-contamination. A reported method for applying coatings containing antimicrobial N-halamines is layer-by-layer (LbL) application of polyelectrolytes, which form N-halamines upon cross-linking. Prior reports on dip layer-by-layer (LbL) fabrication have demonstrated the potential of this coating technology; however, spray LbL fabrication would enable more rapid coating and represents a more commercially translatable application technique. In this work, dip and spray LbL methods were used to coat polypropylene (PP) with N-halamine containing bilayers consisting of cross-linked polyethylenimine (PEI) and poly(acrylic acid) (PAA). Further experimentation with spray LbL fabrication used naturally occurring polyelectrolytes, chitosan and alginate. Materials were characterized using atomic force microscopy (AFM), ellipsometry, contact angle, fourier transform infrared spectroscopy, a chlorine content assay, and a dye assay for amine quantification. All methods of coating application exhibited a 99.999% (5-log) reduction against *Listeria monocytogenes* with application time for spray LbL taking less than 10% of the time required for dip LbL. Spray LbL fabrication of N-halamines is a rapid and inexpensive method to fabricate rechargeable antimicrobial surfaces.

Keywords: Spray layer-by-layer fabrication, N-halamines, antimicrobial coatings

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LIST OF ABBREVIATIONS

LbL – Layer-by-Layer
DI – Deionized Water
PEI – Polyethylenimine
PAA – Poly(acrylic acid)
ATR-FTIR – Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy
AFM – Atomic Force Microscopy
AO7 – Acid Orange 7
DPD – *N*,*N*-diethyl-*p*-phenylenediamine
TSB – Tryptic soy broth
TSA – Tryptic soy agar

CHAPTER 1

INTRODUCTION TO N-HALAMINES AND LAYER-BY-LAYER COATINGS

1.1. Principles and Advantages of N-Halamine

N-halamines are potent antimicrobial moieties that have been shown to be effective at inactivating both Gram negative and Gram positive bacteria (Sun & Sun. 2004). An N-halamine is a compound that contains at least one halogen (chlorine, bromine, or iodine) covalently bonded to nitrogen. Halogenation of an amine, amide, or imide functional group creates an N-halamine. The stability of the covalent bond is highest with an amine group and least stabile with an imide group (Worley & Sun. 1996). The major advantage of N-halamines over other antimicrobial moieties is its ability to regenerate its biocidal effects when exposed to halogens.

N-halamines can be incorporated into inorganic compounds, called chloramines, or into organic compounds in a variety of chemical structures. Chloramines have been used in water treatment applications as a result of having low reactivity with organic matter and high stability. Organic N-halamines can be categorized by a variety of different classes as a result of the numerous compounds that can include a halogen covalently bonded to nitrogen. Hydantoins, N-chlorosuccinimide, Sulfonchloramides, acyclic N-halamines and polymeric N-halamines are all forms of organic N-halamines (Bastarrachea & Goddard. 2013, Worley, et al. 1988). Many cyclic N-halamines are water insoluble and have low toxicity, which makes them highly desirable for water treatment and other aqueous environments (Sun, et al. 1994). In addition to water treatment, organic N-halamines have further application as an antimicrobial surface coating. Compounds containing N-halamines can be grafted onto a surface (Sun & Sun.

2001), deposited via layer-by-layer assembly (alternating layers of polyelectrolytes) (Bastarrachea & Goddard. 2013) or incorporation into electrospun nanofibers (Tan & Obendorf. 2007). N-halamines in antimicrobial coatings have numerous applications in biomedical and food processing industry.

Though N-halamines have been researched for the past decade, their mechanism of microbial inactivation is still somewhat unknown. The halogen is believed to be able inactivate bacteria through dissociation from the nitrogen and the subsequent oxidation of vital structures in the cell membrane (Worley & Sun. 1996, Kenawy, et al. 2007). Once depleted of its halogens, the N-halamine can be refreshed with a dilute solution of halogens, such as household bleach, to restore its antibacterial activity (Kocer, et al. 2011). Chlorine is the most stable of the halogens and therefore is often used for halogenation of N-halamines (Hui & Debiemme-Chouvy. 2013). Iodine is the least stable of the halogens, and is rarely used to create N-halamines, its large atomic radius causes a weaker bond with the nitrogen atom due to less bond overlap (Worley, et al. 1988).

In addition to the type of halogen, the stability and antimicrobial activity of N-halamines can also depend on the pH, temperature, and presence of organic matter (Worley, et al. 1988). Organic matter can interfere with the effectiveness of many N-halamines therefore highly soiled surfaces are not an ideal application. Another source of instability can be repeated exposure to highly levels of halogens. High concentrations of halogens can cause the N-halamine moieties to separate from their parent compound and therefore cause N-halamine coatings to loose their effectiveness over time. Fortunately, food processing plants are only able to sanitize with a maximum concentration of 200

ppm of chlorine, which has been shown to be effective as forming N-halamines as well as preserving the rechargeable nature of the moieties (Bastarrachea, et al. 2014).

Creating surfaces modified with N-halamines is straightforward and inexpensive in comparison to many other antimicrobials, such as silver. Additionally, they have been shown to have long-term stability in both aqueous and non-aqueous environments. N-halamines are inexpensive to maintain, as they are easily refreshable with a dilute concentration of sodium hypochlorite, and are less corrosive than bleach (Hui & Debiemme-Chouvy. 2013). N-halamine compounds and surfaces have demonstrated promising long-term antibacterial effectiveness and therefore have the potential to be successful at reducing microbial loads in the food processing industry.

1.2. Layer-by-Layer Coatings

1.2.1. Background and Principles

Layer-by-layer deposition (LbL) is a coating technique in which alternating layers of oppositely charged species – usually polyelectrolytes – are deposited on a solid support to create thin functional films (Ratner, et al. 2012). A charged surface (or a surface which has undergone initial functionalization by previously mentioned methods) is exposed to the polyelectrolyte of opposite charge. After this first layer binds to the charge surface, the surface is rinsed to remove any extra polymer to prevent uneven layers or weak bonding. After rinsing, the surface is exposed to the second, oppositely charged, polyelectrolyte, which electrostatically interacts with the first layer. Another rinsing step is necessary before the process is repeated with the first polyelectrolyte (Decher, et al. 1998). A layer of interacting oppositely charged polyelectrolytes is called

a bilayer. This process can be repeated to form any number of desired bilayers. The films generated from LbL depositions are relatively thin, around a few nanometers to a few micrometers thick depending on number of bilayers the pH, and the concentration of the polyelectrolyte solutions (Michel, et al. 2005).

Formation of the bilayers can be an issue if many factors are not closely regulated. The selection and control of pH and polyelectrolyte concentration is critical to ensure uniform deposition (Ratner, et al. 2012). The stability of layer-by-layer deposited coatings is due to numerous non-covalent electrostatic and Van der waals interactions, and can be improved by covalent cross-linking to enhance stability.

1.2.2. Methods of Layer-by-Layer Coating

The modified surface is usually submerged into polyelectrolyte solutions to form these layers – called dip LbL assembly. However, this can be time consuming, require a large amount of polymer solutions, and is difficult for large and/or non-planar surfaces.

Layer-by-layer deposition can also be accomplished using spin coating to create each layer and rinse in between layers. The thickness of the bilayer in spin LbL can be controlled by spin speed, spin time, solvent evaporation rates, and the concentrations of the polyectrolyte solutions (Jiang, et al. 2004). A major advantage of spin LbL assembly over the traditional dip LbL is that it can create bilayers more quickly (reducing time from around 30 minute per bilayer to 2 minutes (Jiang, et al. 2004). However, it is limited in that only one side can be modified at a time (Cho, et al. 2001) and would only be applicable for smaller, planar surfaces.

Alternatively, the layers can be sprayed onto the modified surface, which is a more rapid method and better suited for uneven or large surfaces. It has been reported

that spray layer-by-layer creates homogenous films with 1/25th the contact time of dip layer-by-layer assembly (Izquierdo, et al. 2005). Weak to strong polyectrolyte solutions, solutions that are able to form hydrogen bonds, and even colloidal nanoparticles can be used in spray LbL (Nogueira, et al. 2011). Additionally, spray LbL films have been shown to create even layers with less agglomeration and comparable quality to dip LbL films as a result of the quick deposition times (Krogman, et al. 2007, Michel, et al. 2005). Similar to Spin LbL, one side of a surface can be modified at a time with Spray LbL unless multiple nozzles are fitted to coat both sides.

Spray, spin and dip LbL methods each have their own advantages and disadvantages, however, the resulting coating for all methods can be used for numerous applications.

1.2.3 Antimicrobial Applications of Layer-by-Layer Deposition

Layer-by-layer coatings can be found in many different applications such as in increasing performance of solar cells (Wang, et al. 2011), stimulating tissue growth on implants (Macdonald, et al. 2011), and creating site specific drug delivery systems (Ariga, et al. 2011). Additionally, antimicrobial agents can be incorporated within the bilayers such as silver nanoparticles (Dubas, et al. 2006), chitosan (Gomes, et al. 2013), and N-halamines (Cerkez, et al. 2011, Bastarrachea & Goddard. 2013). When small antimicrobials are trapped in between bilayers, they usually must migrate from the material to inactive bacteria. Therefore, over time their effectiveness will be limited in a food processing environment as the antimicrobial component becomes depleted. However, if the antimicrobial component could be replenished, such as re-halogenating N-halamines, then this issue could be mitigated.

N-halamines can be incorporated into LbL depositions by being apart of a compound trapped between bilayers, in one of the polyelectrolytes or from a bound formed by the polyelectrolytes (Bastarrachea, et al. 2014). After the bilayers are deposited, the coating can be exposed to an aqueous solution of halogens to regenerate the N-halamines (Bastarrachea & Goddard. 2013). The modified coating is then able inactivate various bacteria that comes into contact with its surface. Once the halogens become depleted, it can be refreshed and regenerate its antimicrobial activity by being exposed to a solution of chlorine, bromine, or iodine.

Overall, layer-by-layer assembly is a rapid, cost-effective method to create antimicrobial coatings on a wide variety of surfaces in food processing environments.

CHAPTER 2

DIP AND SPRAY LAYER-BY-LAYER DEPOSITION OF ANTIMICROBIAL N-HALAMINES [11]

2.1. Introduction

Reducing microbial cross-contamination remains an ongoing challenge for the food industry. An estimated 48 million people are affected by foodborne illness each year in the United States. Of those 48 million people, 128,000 are hospitalized, and 3,000 die as a result of contaminated food (Centers for Disease Control and Prevention. 2011). Despite the implementation of well-established cleaning and sanitization protocols of food contact materials used in processing and handling, cross-contamination remains a significant challenge to food safety and quality. Effective sanitation of non-planar food contact surfaces (e.g. corrugated, sharp bends) is a particular challenge as these areas are often not properly cleaned (Austin & Bergeron. 1995). Application of antimicrobial coatings on food contact materials (e.g. plastic or stainless steel) in food processing environments may help reduce survival and cross-contamination of microorganisms in between sanitization cycles and may help to prevent biofilm formation (Luo, et al. 2006).

A number of antimicrobial coatings have been reported in the literature. A major advantage to antimicrobial coatings in which N-halamines are the antimicrobial agent is their ability to regenerate activity with each exposure to a halogen solution. Further, they have shown effectiveness against a broad spectrum of bacteria (Sun & Sun. 2004, Hui & Debiemme-Chouvy. 2013). N-halamines are antimicrobial moieties in which a

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halogen is covalently bound to the nitrogen of an amine, amide, or imide (Worley & Sun. 1996). While their exact mechanism of action has not been fully agreed upon, N-halamines are believed to reduce microbial loads by oxidizing vital cellular membrane structures (Worley & Sun. 1996, Kenawy, et al. 2007). N-halamines can be incorporated into antimicrobial materials via methods including surface grafting (Sun & Sun. 2001), electrospun nanofibers (Tan & Obendorf. 2007), or layer-by-layer assembly, in which alternating layers of oppositely charged polyelectrolytes are deposited onto a surface (Ratner. 1995, Cerkez, et al. 2011). Dip LbL deposition is a well-established fabrication method in which the surface is alternately submerged in oppositely charged polyelectrolyte solutions, with a wash step in between each solution. This cycle can be repeated until the desired number of bilayers is formed. An alternative method, spray LbL deposition (**Figure 2.1**), coats surfaces with aerosolized droplets of the polyelectrolyte solution instead of submerging the surface in a solution of the polyelectrolyte(Nogueira, et al. 2011). Major benefits of spray deposition of LbL coatings are the ability to coat large, irregular pieces uniformly and the significantly (> 90%) reduced time required for application. Further, the spray method utilizes less solution and therefore generates less waste, which is both cost effective and beneficial for the environment (Izquierdo, et al. 2005). These characteristics make spray LbL an attractive coatings technology in terms of commercial adoption.

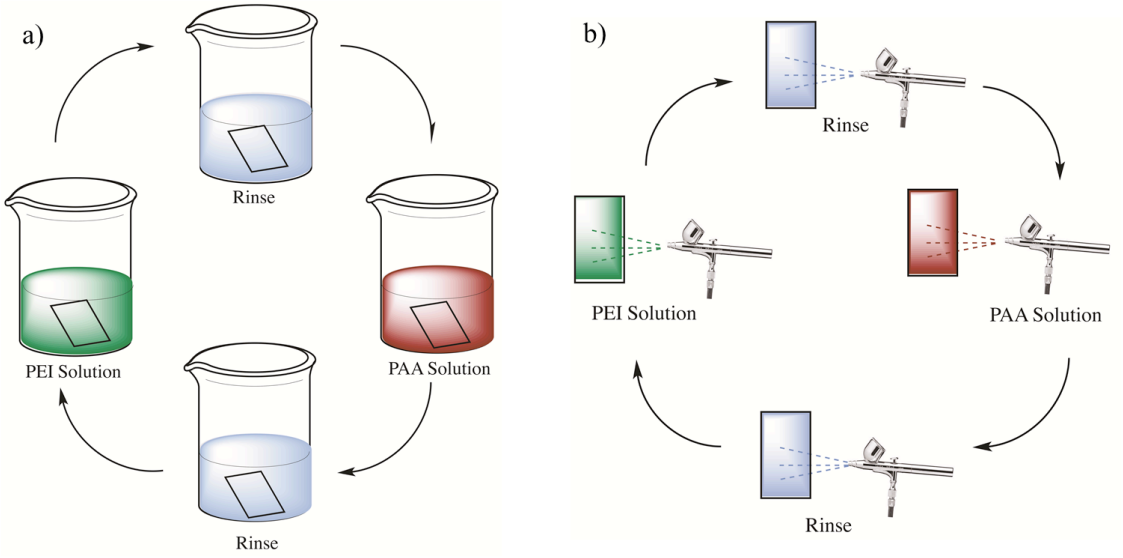

Figure 2.1. Comparison of the a) dip layer-by-layer b) spray layer-by-layer assembly methods

Previous work has demonstrated that LbL deposition of PEI and PAA by dip LbL assembly can enable more than a 5 logarithmic reduction in the initial bacteria population(Bastarrachea, et al. 2014). We hypothesized that preparation of N-halamine coatings via spray LbL assembly would result in a similar bacterial reduction with a fraction of the fabrication time of dip LbL assembly. The goal of this study was therefore to compare spray LbL deposition against dip LbL deposition in terms of chlorine regenerability and effectiveness in inactivating *Listeria monocytogenes*.

2.2 Materials and Methods

2.2.1 Preparation of Films and Silicon Wafers

Two solid supports were used in this work: polypropylene films to enable parallel

comparison with prior work and to represent a commercially relevant material, and silicon wafers to facilitate quantitative measurements via ellipsometry and atomic force microscopy. Polypropylene pellets (PP, Scientific Polymer Products, Ontario, NY) were pressed into thin films of 150 μm thickness on a hot press. The films were cut into 1 × 1 cm squares for the dip LbL treatment and into 6 × 10 cm rectangles for the spray treatments. Silicon wafers were diced into 1 × 2 cm rectangles. The films and silicon wafers were then cleaned using sonication at 40 kHz, starting with two cycles of isopropanol (Fisher Scientific, Fair Lawn, NJ), then two cycles of acetone (Fisher Scientific, Fair Lawn, NJ), and lastly with deionized (DI) water. The PP films and silicon wafers were dried overnight over anhydrous calcium sulfate with a relative humidity of less than 20%.

PP films and Si wafers were functionalized prior to LbL deposition to improve adhesion of N-halamine coating. Each side of each PP film was exposed to UV irradiation for 15 min with a Jelight Co. model 42 UVO Cleaner (Irvine, CA) to generate amine-reactive carboxylic acid groups. Silicon wafers were cleaned and oxidized with piranha solution (30% hydrogen peroxide (Fischer Scientific, Fair Lawn, NJ) and 70% sulfuric acid (Fisher Scientific, Fair Lawn, NJ)) followed by rinsing in copious water. Piranha treated wafers were then submerged in a 2% solution of 3-glycidyloxypropyltrimethoxysilane (GOPTS, Acros Organics, Fair Lawn, NJ) in toluene ((Fisher Scientific, Fair Lawn, NJ) for 10 min followed by rinsing in toluene to introduce amine-reactive epoxy groups. Epoxy-functionalized silicon wafers were cured at 80 °C for 2 h to ensure cross-linking of the silane monolayer.

The following solutions were prepared for use in both dip and spray LbL

treatments: 0.1% (w/w) branched polyethylenimine (PEI, Sigma Aldrich, St. Louis, MO) in DI water, adjusted to pH 10.0 with 1.0 M sodium hydroxide; 0.1% (w/w) poly(acrylic acid) (PAA, Scientific Polymer Products, Ontario, NY) in DI water, adjusted to pH 4.0 with 1.0 M hydrochloric acid; 0.1 M 2-(N-Morpholino)ethanesulfonic acid (MES, GenScript Piscataway, NJ) buffer, pH 5.2, to which 5 mM 1-(3-Dimethyloaminopropyl)-3-ethylcarbodimmide hydrochloride (EDC-HCl, ProteoChem, Denver, CO) and 0.5 mM N-hydrosuccinimide (NHS, Acros Organics, Fair Lawn, NJ) were added immediately prior to each cross-linking treatment.

Three LbL fabrication methods were explored in this and are referred to as follows: “Dip/Dip” in which LbL application and cross-linking were both conducted by full submersion of coupons in reagents; “Spray/Dip” in which LbL application was by spraying polyelectrolytes and cross-linking was performed by full submersion in cross-linking reagent; and “Spray/Spray” in which both LbL application and cross-linking reagents were applied to the coupons by spraying.

For Dip/Dip LbL fabrication, coupons (PP or Si) were immersed in PEI solution (4 mL/ coupon) and stirred for 5 min followed by rinsing in copious DI water. Coupons were then transferred to another beaker that contained PAA solution (4 mL/coupon) and stirred for 5 min. After another washing step the coupons were returned to a beaker filled with PEI solution for application of subsequent bilayers. A total of 10 bilayers was applied before submersion in cross-linker solution (MES buffer containing EDC and NHS) for 2 h, rinsing, and drying overnight over anhydrous calcium sulfate. The chemistry of the applied coating is illustrated in **Figure 2.2**.

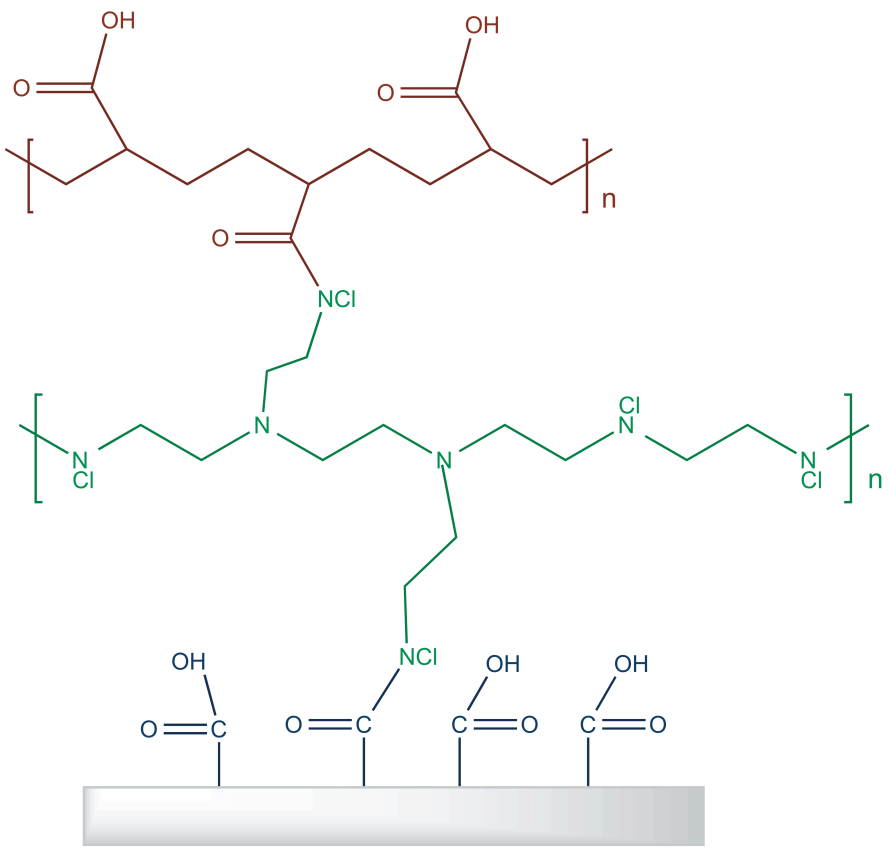

Figure 2.2. Surface chemistry changes after UV-Ozone irradiation of polypropylene (PP) and cross-linking of 1-(3-Dimethyloaminopropyl)-3-ethylcarbodimmide hydrochloride (EDC-HCl) and N-hydrosuccinimide (NHS) of polyethylenimine (PEI) and poly(acrylic acid) (PAA) adapted from *Bastarrachea et al.* (2013).

For Spray/Dip LbL fabrication, coupons were mounted to a vertical support to allow excess solution to drain off the films. PEI solution was sprayed onto the coupons for 10 s at a distance of 15 cm with a pressure of 40 psi and a nozzle tip diameter of 0.4 mm, followed by spraying for 30 s with DI water. Then the coupons were sprayed with PAA solution for 10 s followed by spraying for 30s with DI water. After application of 10 bilayers, coupons were rinsed again and submerged in cross-linker solution (MES buffer with 5 mM EDC-HCl and 0.5 mM NHS) for 2 h. N-halamine modified coupons

were then rinsed three times with DI water and dried over anhydrous calcium sulfate overnight.

For Spray/Spray LbL fabrication, bilayers were applied as described for Spray/Dip LbL fabrication, however instead of submerging the coupons in the cross-linker solution, the cross-linkers EDC-HCl and NHS were included in the PEI solution at concentrations of 5 mM and 0.5 mM, respectively.

2.2.2 Characterization of N-halamine Coatings

The surface morphology and roughness of clean and N-halamine modified silicon wafers were analyzed using a Dimension 3100 Atomic Force Microscope (Digital Instruments, Santa Barbara, CA) under tapping mode with a Si N-type tip (uncoated, f_0: 200 – 400 kHz, Applied NanoStructures, Inc., Mountain View, CA). As noted above, silicon wafers were used to enable quantitative roughness and thickness measurements. One measurement was taken from a random spot on three independent pieces for all treatments per each replicate. These resulting images were analyzed through the SPIP software version 6.0.6 (Scanning Probe Image Processor, Image Metrology, Denmark). The SPIP software used to calculate mean square roughness (S_q).

Thickness of applied coatings was quantified using an SL-II automatic ellipsometer (Rudolph Research Analytical, Hackettstown, NJ). The ellipsometer was used with an angle of incidence of 70° from the normal and a He-Ne laser with wavelength of 632.8 nm. Three different spots were analyzed per each wafer and three wafers were analyzed per each replicate.

The hydrophobicity of native and modified coupons was characterized using advancing and receding water contact angles on a DSA100 (Krüss, Hamburg, Germany).

A volume of 5 μL DI water was dispensed from an automatic syringe at a rate of 25 μL/min. Images were taken and measurements were analyzed using the Drop Shape Analysis software, dynamic sessile drop method, version 1.91.0.2 (Krüss, Hamburg, Germany). Hysteresis was obtained from the difference between advancing and receding water contact angles. Four measurements from different coupons were taken for each treatment.

Surface chemistry of native and modified polypropylene coupons was characterized using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and a dye assay. ATR-FTIR spectra were measured using the Prestige 21 spectrometer (Shimadzu Corp., Tokyo, Japan) equipped with a diamond ATR crystal. For each replicate the absorbance was measured for five coupons in two different places per each coupon. A total of 32 scans were applied for every measurement using Happ-Genzel apodization and a resolution of 4 cm^{-1}. Spectra were acquired from the IRsolution software (Shimadzu Corp., Tokyo, Japan) and analyzed with the KnowItAll software (Biorad Laboratories, Philadelphia, PA).

A colorimetric dye assay was used to determine the primary amine content of the coupons (Uchida, et al. 1993). Each 1 × 1 cm coupon was shaken in 1 mM Acid Orange 7 (AO7) dye (Orange (II), Acros Organics, Fair Lawn, NJ) in DI water adjusted to pH 3.0 by 1.0 M hydrochloric acid for 3 h to allow dye to complex with primary amines on the coupons. After rinsing in DI water at pH 3.0 to remove adsorbed dye, coupons were shaken for 15 min in DI water adjusted to pH 12.0 with 1.0 M sodium hydroxide to disassociate dye. Absorbances were measured at 455 nm and calculated as $nmol/cm^2$ primary amines by comparison to a standard curve of AO7 in DI water at a pH of 12.0.

Dye assays were performed in two replicates with four samples per assay.

The number of N-halamines present on modified coupons was quantified using a N,N-diethyl-p-phenylenediamine (DPD) assay. Amines and amides present in the N-halamine coating were first chlorinated by exposure to 200 ppm sodium hypochlorite (Acros Organics, Fair Lawn, NJ) for 30 min. Initial chlorine content of the stock sodium hypochlorite was quantified by idometric titration with 0.1 N sodium thiosulfate (American Society for Testing and Materials. 2008). After chlorination, coupons were rinsed in excess water then placed in individual test tubes to which 2 mL DI water and 50 μL of DPD reagent (prepared by mixing a foil packet of DPD total chlorine reagent powder (Hach, Loveland, CO) with 1 mL of DI water) were added. The tubes were shaken for 5 min to allow for color formation. Absorbances were measured at 512 nm. N-halamine content of each coupon was calculated by comparison to a standard curve prepared from sodium hypochlorite in water. Assays were performed in two replicates with four samples tested per assay.

2.2.3 Antimicrobial Activity

The antimicrobial activity of the N-halamine coated coupons was demonstrated against *Listeria monocytogenes* Scott A (provided by Dr. Martin Wiedmann, Cornell University, Ithaca, N.Y.). An isolated colony of *L. monocytogenes* was incubated at 37 °C for 14 h in tryptic soy broth (TSB, Becton, Dickinson and Company Sparks, MD). A 1:100 dilution was made from this suspension and incubated at 37°C for 4 h, after which a 1:1000 dilution was made with DI water. An aliquot of this dilution was plated onto tryptic soy agar (TSA, Becton, Dickinson and Company Sparks, MD) to determine the initial concentration of bacteria. Coupons were submerged in 1 mL aqueous suspension

to a final surface area:volume ratio of 6 cm^2/mL and incubated for 2 h at 32° C with rotation at 60 rpm. Suspensions from each tube were diluted to 10% with neutralizing buffer (Becton, Dickinson and Company Sparks, MD), to quench the chlorine, and then plated onto TSA in two replicates. The plates were incubated for 48 h at 37° C and counted using a plate reader Scan 500 (Interscience, Saint-Non-la-Brèteche, France). Inactivation tests were repeated four times on different days.

2.2.4 Statistical Analysis

To determine statistical significance between treatments Analysis of Variance and Tukey's pairwise comparison was applied, when appropriate, with a 95% confidence interval using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA).

2.3 Results and Discussion

2.3.1 Surface Morphology and Coating Thickness

Surface morphologies of silicon modified by GOPTS silanization and the LbL assembled coating techniques (Dip/Dip, Spray/Dip, and Spray/Spray) were characterized using AFM (**Figure 2.3**). The GOPTS silanized silicon exhibited low roughness values with an S_a of 0.55 ± 0.03 nm and S_q of 1.924 ± 0.079 nm (**Table 2.1**). Spray/Dip and Spray/Spray treatments show similar surface topography on the scale of ± 300 nm with significantly different ($P < 0.05$) roughness values (S_q of 125.8 ± 16.9 nm and 84.88 ± 5.28 nm for Spray/Dip and Spray/Spray, respectively). However, the Dip/Dip LbL treatment shows a much greater range in surface roughness, ± 800 nm with roughness value of 286.5 ± 6.82 nm. Previous studies have shown that longer contact time with the polyelectrolytes in dip LbL can cause the formation of "islands" or clumps of the

polyelectrolyte leading to uneven deposition (Guzmán, et al. 2011, Krogman, et al. 2007). However, both spray LbL assembly methods were exposed to the polyelectrolyte for 10 s before a rinse step and therefore would not have formed "islands" of polyelectrolytes resulting in decreased surface roughness.

The thickness of the LbL deposited coating for silanized silicon, Dip/Dip LbL, Spray/Dip LbL, and Spray/Spray on silanized silicon wafers were measured using spectroscopic ellipsometry. The thickness of GOPTS was 4.93 ± 0.82 nm (**Table 2.1**). The coating for the Spray/Dip LbL sample, which was submerged in cross-linker solution for 2 h, was significantly thicker (394 ± 26 nm) than the Spray/Spray LbL (238 ± 21 nm) ($P < 0.05$). Coupons prepared by Dip/Dip LbL application were non-reflective and therefore could not be characterized by ellipsometry. However, previous research on spray and dip LbL fabrication has shown that dip LbL coatings are often thicker than spray coatings (Kolasinska, et al. 2008, Izquierdo, et al. 2005).

Table 2.1. Surface Roughness and Thickness of Silicon Wafers

Treatment	S_q (nm)	Thickness (nm)
Silanized Silicon Wafer	1.924 ± 0.079^{a}	4.93 ± 0.82^{a}
Dip/Dip	286.5 ± 6.82^{b}	N/A
Spray/Dip	125.8 ± 16.9^{c}	394 ± 26^{b}
Spray/Spray	84.88 ± 5.28^{d}	238 ± 21^{c}

*Treatments with the same letter within the same column are not significantly different, n=6 ($P > 0.05$). Sensitivity of ellipsometer limits thickness data to two significant figures.

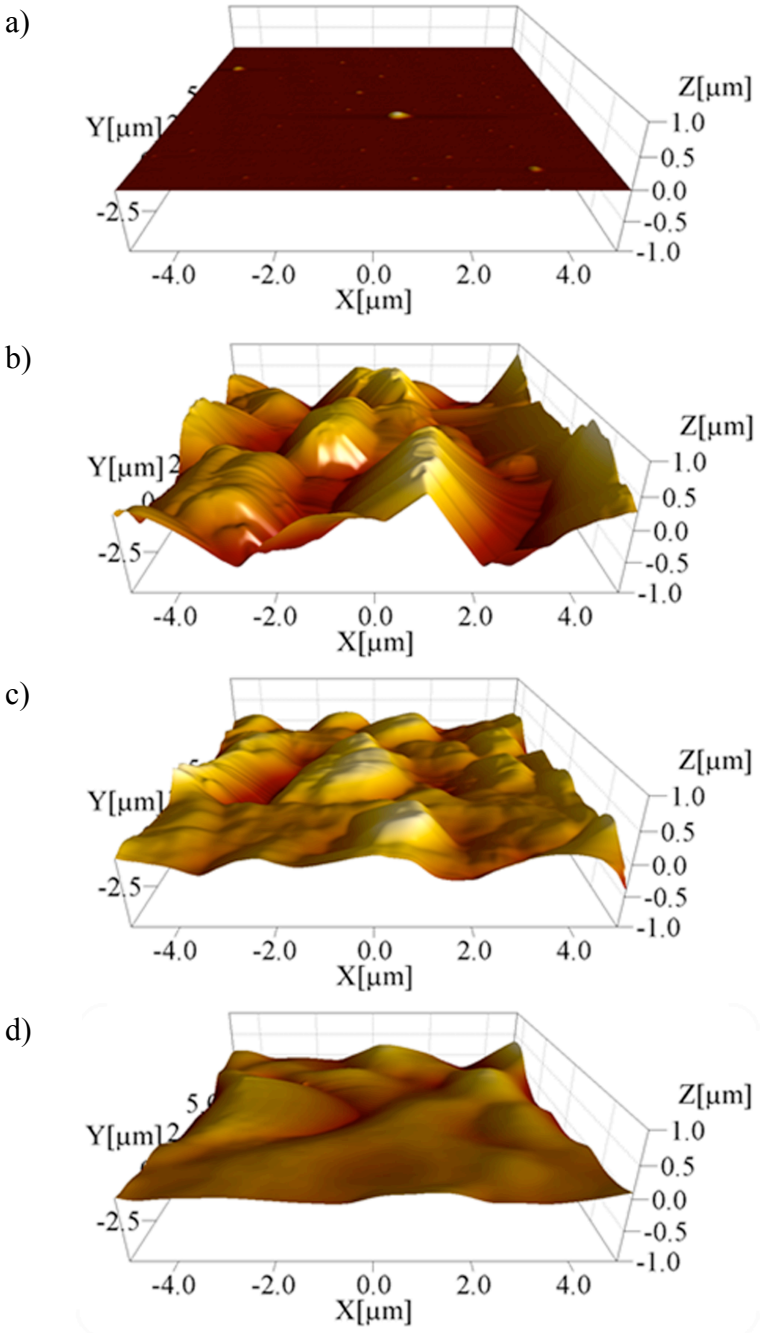

Figure 2.3. Surface characterization of a) silanized silicon b) Dip/Dip and c) Spray/Dip d) Spray/Spray using AFM.

2.3.2 Surface Hydrophobicity

The advancing, receding, and hysteresis (θ_A, θ_R and H, respectively) contact angles were measured for native and coated PP coupons to quantify the effect of LbL deposition on hydrophobicity. Native PP presented a characteristic hydrophobic surface with advancing contact angle of 103.3± 1.5°, in agreement with prior reports (Tian, et al. 2015). Modification with UV-ozone and subsequent deposition of bilayers increased the hydrophilicity of the material. No significant difference ($P > 0.05$) was observed between contact angles of the different LbL application methods, which presented advancing contact angles of 72.2 ± 2.1° to 75.2 ± 1.5 °. These results suggest that each application method yielded similar surface chemistry.

Table 2.2. Advancing and Receding Contact Angle and Contact Angle Hysteresis

Treatment	θ_A (degrees)	θ_R (degrees)	θ_H (degrees)
Native PP	103.3 ± 1.5^{a}	78.1 ± 2.0^{a}	25.3 ± 3.3^{a}
UV-O_3 PP	82.8 ± 2.8^{b}	44.2 ± 1.4^{b}	38.6 ± 3.4^{b}
Dip/Dip	75.2 ± 1.5^{c}	38.8 ± 2.7^{c}	36.4 ± 2.8^{b}
Spray/Dip	73.3 ± 1.4^{c}	33.7 ± 1.9^{cd}	39.6 ± 2.8^{b}
Spray/Spray	72.2 ± 2.1^{c}	32.4 ± 3.6^{d}	39.9 ± 1.6^{b}

*Values are means of three replicates. Treatments with the same letter within the same column are not significantly different ($P > 0.05$).

2.3.3 Surface Chemistry

ATR-FTIR spectroscopy was used to characterize surface chemistry of native and N-halamine modified PP coupons (**Figure 2.4**). Native PP presented absorbances at 2960 – 2850 cm^{-1} and 1465 – 1440 cm^{-1} characteristic of methyl and alkane groups. After UV-ozone treatment, a carbonyl absorbance band was evident in the 1740 – 1720 cm^{-1} range,

indicating successful surface oxidation by UV-ozone and introduction of carbonyl functionality. Absorbance bands characteristic of primary and secondary amines (1650 – 1590 cm^{-1}), amide bonds (1570 – 1515 cm^{-1} for N–H bond and 1680-1630 cm^{-1} for C=O stretch), and hydroxyl groups (1410 – 1390 cm^{-1} and 3400 – 3200 cm^{-1}) were evident in all N-halamine coated PP coupons. Spectra of Spray/Dip and Spray/Spray LbL samples were nearly identical, suggesting that the method of polyelectrolyte deposition had more of an effect on surface chemistry than the cross-linking method. Dip/Dip samples had similar absorbances to the Spray methods, but had notably reduced band intensity for amide and hydroxyl absorbances. These results suggest that more polymers are covalently attaching to the surface in the spray treatments. The formation of amide bonds in all samples indicated successful cross-linking between PEI and PAA. Primary amines have been reported to form a stronger N-halamine chlorine complex than amides or imides (Worley & Sun. 1996).

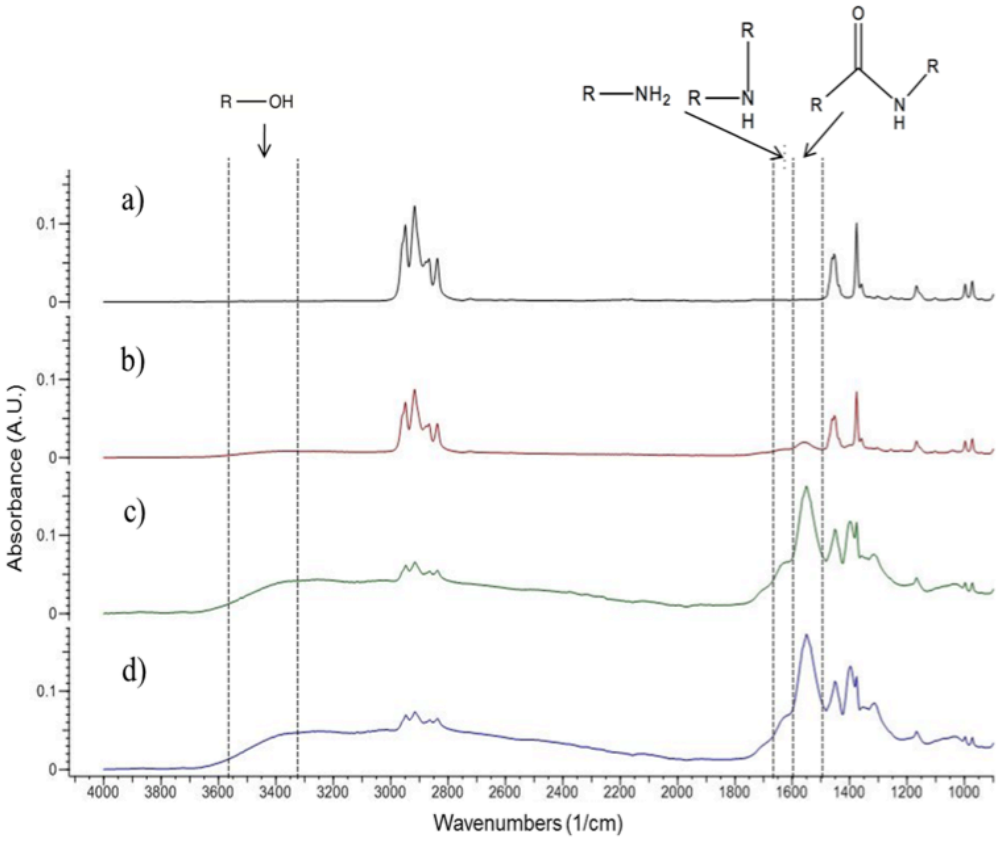

Figure 2.4. FTIR Spectra a) native polypropylene b) Dip/Dip, c) Spray/Dip, and d) Spray/Spray

A dye assay, acid orange 7, was used to quantify primary amine content of the coatings (**Figure 2.5**). Both Spray application methods resulted in significantly higher primary amine contents compared to the Dip application method, with no significant difference between the two Spray application methods. These results are in support of FTIR interpretations, which suggested that Spray/Spray and Spray/Dip yielded similar surface chemistries. Therefore, chlorine content measurements and antimicrobial activity tests were completed for only the Dip/Dip and Spray/Spray LbL samples.

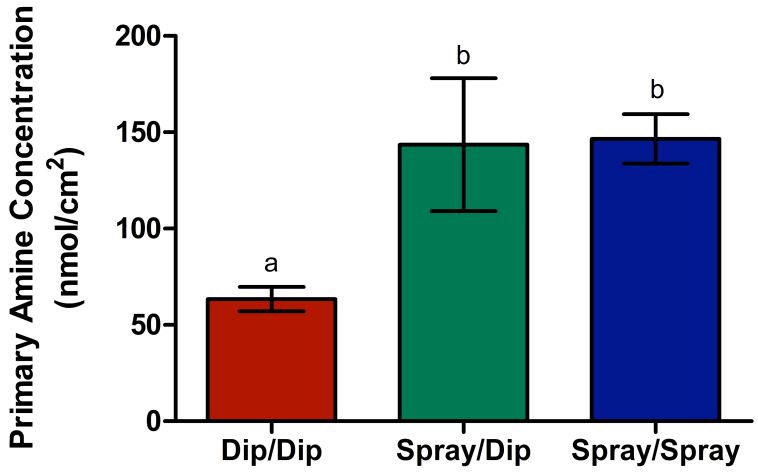

Figure 2.5. Primary amine content of Dip/Dip, Spray/Dip, and Spray/Spray determined by Acid Orange 7 Dye Assay. Data shown are averages from two replicate assays (n=8). Treatments followed by the same letters are not significantly different ($P > 0.05$).

The chlorine content of native and N-halamine coated PP coupons was quantified using a DPD assay (**Figure 2.6**). There was no significant difference between chlorine contents of the Dip/Dip and Spray/Spray samples (~50 nmol/cm^2, $P > 0.05$). Extended exposure of the N-halamine coated PP coupons to 200 ppm sodium hypochlorite (300 total minutes, equivalent to 300 sanitization cycles in a food production facility (U.S. Food and Drug Administration. 2014), reduced N-halamine content by approximately 90%. In prior work, a similar Dip LbL application method retained N-halamine content for up to 25 cleaning cycles (Bastarrachea, et al. 2014). The observed reduction in N-halamine content in this work may be a result of delamination due to differences in cross-linking as well as the length of chlorine exposure. It is likely that the cross-linking method is causing the delamination as the cross-linkers promote the formation of amides, which form less stable N-halamines than amines that are originally present in the PEI (Tan & Obendorf. 2007). Futhermore, amides, unlike amines, hydrolyze due to exposure

to the alkaline pHs, such as sodium hypochlorite, leading to delamination (Robinson & Tester. 1990). On-going work is investigating alternative (less alkaline) halogenated sanitizers for rechlorination of N-halamine coatings.

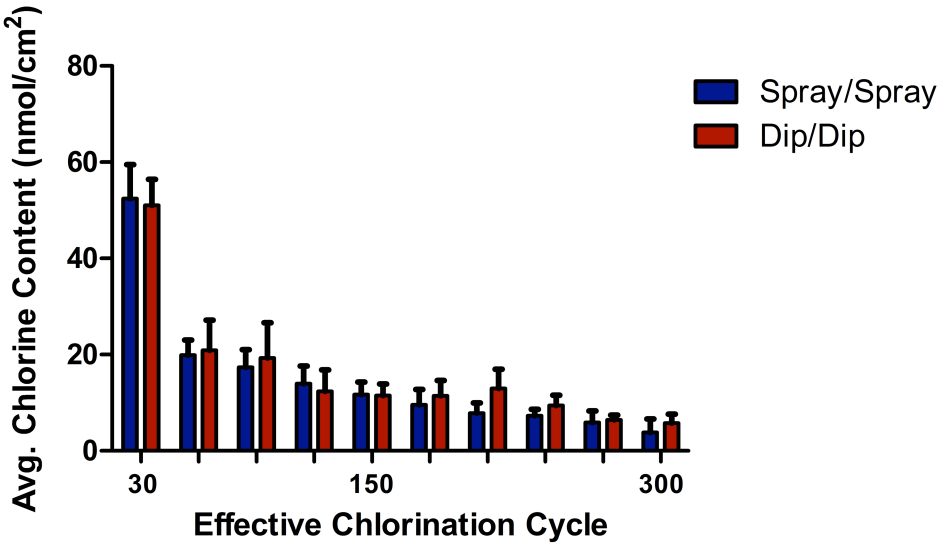

Figure 2.6. Chlorine holding capacity and regeneration ability of Dip/Dip and Spray/Spray over 10 chlorination cycles of 200 ppm chlorine for 30 min per cycle with subsequent quenching in between cycles. Data shown are averages from two replicate assays (n=8).

2.3.4 Antimicrobial Activity

Native and N-halamine modified PP coupons were chlorinated then submerged in aqueous suspensions of *L. monocytogenes* (started concentration of ~ 5 log (CFU/mL) to characterize their antimicrobial activities. After 2 h incubation at 32°C, chlorinated native PP presented an average bacterial population of 6.19 log (CFU/mL). Both Dip/Dip and Spray/Spray application methods yielded a greater than 5 logarithmic microbial reduction (> 99.999%) compared to native PP and bacterial suspension alone (**Figure 2.7**). These results suggest that the observed antimicrobial activity is derived

from the N-halamine coating. Both Dip/Dip and Spray/Spray LbL treatments obtained an inactivation that exceeded that limit of detection, which is less than 1 log (CFU/mL).

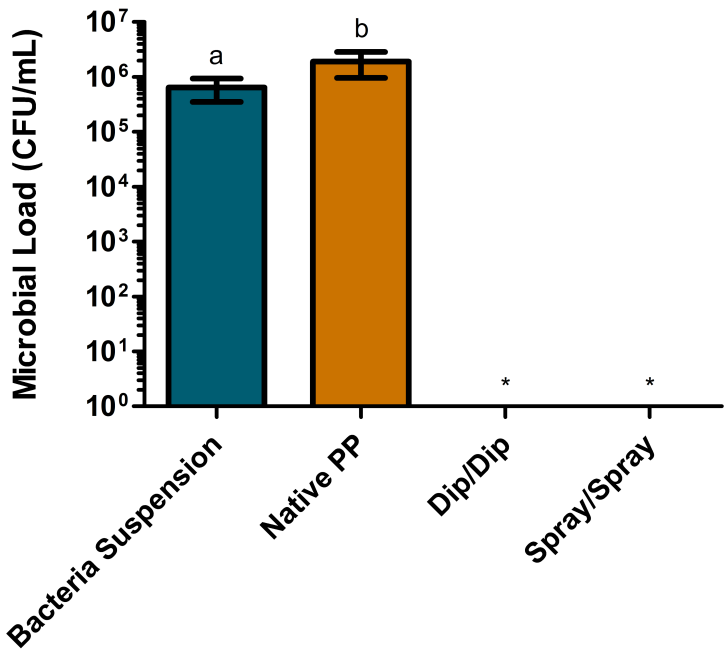

Figure 2.7. Antimicrobial activity of a) Dip/Dip b) Spray/Spray against *Listeria monocytogenes* after 2 hours of contact with samples at 32°C (n=4). *Both dip and spray treatments were below the limit of detection (< 1 log (CFU/mL)).

2.4 Conclusion

Refreshable antimicrobial coatings, such as those containing N-halamines, represent a promising method to reduce microbial cross-contamination in food processing environments. In this work, spray and dip layer-by-layer application methods, were compared and characterized using surface morphology, surface hydrophobicity, surface chemistry, and antimicrobial activity against *L. monocytogenes*. Both deposition methods, spray and dip LbL assembly, were able to achieve a reduction in the population of *L. monocytogenes* of > 5 logarithmic cycles or > 99.999%. Surface modification of the

materials was confirmed using ATR-FTIR and by quantifying the surface concentration of primary amines. Changes in surface morphology, observed through using AFM and dynamic contact angle, further supported evidence of a modification. The stability of the N-halamine coating was evaluated through multiple chlorination and wash cycles. The coating began to show significant delamination after the equivalent of 300 sanitation cycles, which could be resulting from hydrolysis of the amides due to the alkaline pH of sodium hypochlorite. Further work is underway to optimize the cross-linking process to maintain regenerability. Spray LbL deposition of N-halamines is a rapid fabrication method with comparable antimicrobial effectiveness to dip LbL deposition at <10% the fabrication time, supporting its commercial translatability.

CHAPTER 3

SPRAY LAYER-BY-LAYER DEPOSITION OF ANTIMICROBIAL N-HALAMINE WITH CHITOSAN AND ALGINATE

3.1 Introduction

Many consumers are rejecting synthetic additives and processing agents in favor of natural, more environmentally conscious alternatives (Devcich, et al. 2007). However, control of bacteria is crucial in a food processing plant, as contamination from contact surfaces to food products can lead to contamination with spoilage and pathogenic bacteria. As a result, food companies looking to appeal to environmentally concerned consumers may opt for greener control of microbes, such as naturally sourced antimicrobial coatings. Antimicrobial coatings in a food processing plant could effectively reduce microbial loads in between sanitization cycles and increase the safety and shelf life of our food.

N-halamines are effective and rechargeable antimicrobial moieties that can be incorporated onto a multitude of surfaces using layer-by-layer deposition. Spray LbL assembly, which allows aerosolized droplets of the polyelectrolytes to be deposited, represents a rapid fabrication method that utilizes less solution and generates less waste, which is both cost effective and an environmentally conscious choice (Izquierdo, et al. 2005). Previously discussed work in Chapter 2 has demonstrated that LbL deposition of PEI and PAA by spray LbL assembly can enable a greater than a 5 logarithmic reduction in the initial bacteria population of *Listeria monocytogenes*.

Often, the polyelectrolytes used to form the oppositely charged layers in layer-by-layer deposition are synthetic, such as PEI and PAA (Izquierdo, et al. 2005, Dubas, et

al. 2006, Bastarrachea, et al. 2014), however, two naturally derived polyelectrolytes could be used as an alternative. We hypothesized that preparation of N-halamine coatings via spray LbL assembly with chitosan and alginate would result in a similar bacterial reduction against pathogenic bacteria, *Listeria monocytogenes*. The polyanion, alginate, is derived from brown algae and the polycation, chitosan, is derived from crustacean shells. Chitosan has been shown to have antimicrobial properties (Devlieghere, et al. 2004) and contains amines that can be formed into antimicrobial N-halamines and cross-linked to the carboxylic acid group of the alginate.

The goal of this study was to use chitosan and alginate to create a spray LbL N-halamine coating that is rechargeable and effective antimicrobial surface.

3.2 Materials and Methods

3.2.1 Spray Layer-by-Layer Fabrication of N-halamine Coatings

Polypropylene pellets (PP, Scientific Polymer Products, Ontario, NY) were pressed into thin films of 150 μm thickness on a hot press. The films were cut into 6 × 5 cm rectangles. The films were cleaned using sonication at 40 kHz, starting with two cycles of isopropanol (Fisher Scientific, Fair Lawn, NJ), then two cycles of acetone (Fisher Scientific, Fair Lawn, NJ), and lastly with deionized (DI) water. The PP films were dried overnight over anhydrous calcium sulfate with a relative humidity of less than 20%.

One side of the PP films underwent UV irradiation for 15 min with a Jelight Co. model 42 UVO Cleaner (Irvine, CA) to generate reactive carbonyl group and improve covalent attachment to primary amines.

A 0.1% (w/w) solution of chitosan (≥ 75% deacetylated, Sigma Aldrich, St. Louis, MO) was prepared in 1% solution of acetic acid (Fischer Scientific, Fair Lawn, NJ) with the pH adjusted to 5 using 1.0 M NaOH. A 0.1% (w/w) solution of alginate (Sigma Aldrich, St. Louis, MO) was prepared in DI water with the pH adjusted to 5 using HCl. A 0.1 M 2-(N-Morpholino)ethanesulfonic acid (MES) sodium salt (GenScript Piscataway, NJ) buffer was prepared, pH 5.2, to which 5 mM 1-(3-Dimethyloaminopropyl)-3-ethylcarbodimmide hydrochloride (EDC-HCl, ProteoChem, Denver, CO) and 0.5 mM N-hydrosuccinimide (NHS, Acros Organics, Fair Lawn, NJ) were added immediately prior to each cross-linking treatment. Films were mounted to a vertical support to allow for excess solution to drain off the films. The chitosan solution was sprayed onto the films for 10 s at a distance of 15 cm with a pressure of 40 psi and a nozzle tip diameter of 0.4 mm. Next, the film was sprayed for 30 s with DI water. The film was sprayed with alginate solution for 10 s and spray for 30 s with DI water. This process was repeated for 5 bilayers. After the 5 bilayers, the films were sprayed for 60 s with cross-linker solution and then sprayed for 30 s with DI water. The film was dried over anhydrous calcium sulfate overnight.

3.2.2 Characterization of N-halamine Coatings

The hydrophobicity of the modified PP was characterized using advancing (θ_A) and receding (θ_R) water contact angles were measured with a DSA100 (Krüss, Hamburg, Germany). A volume of 5 μL DI water was dispensed with an automatic syringe at a rate of 25 μL/min. Images were taken and measurements were analyzed using the Drop Shape Analysis, dynamic sessile drop method, version 1.91.0.2 (Krüss, Hamburg, Germany). Hysteresis (H) was obtained from the difference between advancing and

receding water contact angles. Four measurements from different coupons were taken for each treatment.

Surface chemistry of native and modified polypropylene coupons was characterized using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and a colorimetric dye assay using Acid Orange 7. ATR-FTIR spectra were measured using the Prestige 21 spectrometer (Shimadzu Corp., Tokyo, Japan) equipped with a diamond ATR crystal. For each replicate the absorbance was measured for five coupons in two different places per each coupon. A total of 32 scans will were applied for every measurement using Happ–Genzel apodization and a resolution of 4 cm^{-1}. Spectra were acquired from the IRsolution software (Shimadzu Corp., Tokyo, Japan) and analyzed with the KnowItAll software (Biorad Laboratories, Philadelphia, PA). A colorimetric dye assay was used to determine the primary amine content of the films. Each 1 × 1 cm coupon was shaken in 1 mM of Acid Orange 7 (AO7) dye (Orange (II), Acros Organics, Fair Lawn, NJ) in DI water adjusted to pH 3.0 by 1.0 M hydrochloric acid for 3 h. Coupons were rinsed three times with DI water with a pH of 3.0 to wash away extra dye and transferred to DI water adjusted to pH 12.0 by 1.0 M sodium hydroxide to disassociate the dye. After 15 min, the absorbance of the solutions was measured at 455 nm and calculated average surface concentration ($nmol/cm^2$) by comparison to a standard curve of AO7 dye in DI water adjusted to pH 12. Assays were performed in two replicates with four samples per assay.

The number of N-halamines present on modified coupons was quantified using N,N-diethyl-p-phenylenediamine (DPD) assay. Amines and amides present in the N-halamine coating were first chlorinated by exposure to 1 mL 200 ppm chlorine (Acros

Organics, Fair Lawn, NJ) for 30 min. Initial chlorine content of the stock sodium hypochlorite was quantified by idometric titration with 0.1 N sodium thiosulfate (American Society for Testing and Materials. 2008). After chlorination, 1 × 1 cm coupons were rinsed in excess water then placed in individual test tubes to which 2 mL DI water and 50 μL of DPD reagent (prepared by mixing a foil packet of DPD total chlorine reagent powder (Hach, Loveland, CO) with 1 mL of DI water) were added. The tubes were shaken for 5 min to allow for color formation. Absorbances were measured at 512 nm. N-halamine content of each coupon was calculated by comparison to a standard curve prepared from sodium hypochlorite in water. The DPD assay was repeated for the 1st, 5th, and 10th cycles. For the remaining cycles, the bound chlorine was quenched with 1 mL of 0.1 N sodium thiosulfate for 1 min. Assays were performed in two replicates with four samples tested per assay.

3.2.3 Antimicrobial Activity

The antimicrobial activity of the N-halamine coated coupons was challenged against the Gram positive bacterium *Listeria monocytogenes*. An isolated colony of *L. monocytogenes* was incubated at 37 °C for 14 h in a sterile glass tube containing tryptic soy broth (TSB, Becton, Dickinson and Company Sparks, MD).

A 1% dilution was made from this suspension in a sterile glass tube and incubated (with a glass tube of new TSB) at 37 °C. After 4 h, a 0.1% dilution was made with DI water. An aliquot of this dilution was plated onto tryptic soy agar (TSA, Becton, Dickinson and Company Sparks, MD) and 1 mL of this dilution was transferred into sterile glass tubes containing a total of 6 1 × 1 cm coupons of the modified and native PP, both of which underwent a chlorination step.

Glass tubes were incubated for 2 h at 32 °C. The glass tubes were rotated at a speed of 60 rpm. After 2 h, 10% dilutions were made from the suspension with neutralizing buffer (Becton, Dickinson and Company Sparks, MD) and then plated onto TSA in replicates. The plates were incubated for 48 h at 37 °C and counted using a plate reader Scan 500 (Interscience, Saint-Non-la-Brèteche, France). Inactivation tests were completed in two replicates with four samples per test.

3.2.4 Statistical Analysis

To determine statistical significance between treatments, Analysis of Variance and Tukey's pairwise comparison was applied, when appropriate, with a 95% confidence interval using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA).

3.3 Results and Discussion

3.3.1 Surface Hydrophobicity

The advancing, receding, and hysteresis contact angles were measured for native PP, UV-ozone modified PP, and the chitosan and alginate modified samples to observe the effect of LbL deposition on hydrophobicity. All samples were significantly different ($P < 0.05$) in advancing, receding, and hysteresis contact angles (**Table 3.1**). Native polypropylene is a hydrophobic substance with a measured advancing contact angle of 104.5 ± 1.7° (Tian, et al. 2015). Modification with UV- ozone increased the wettablity, creating a hydrophilic surface (86.0 ± 2.0°), characterized by having an advancing angle of less than 90 degrees. A further decrease in advancing contact angle was observed for the chitosan and alginate LbL sample (72.3 ± 1.7°). An increase in hydrophilicity is

desirable for an antimicrobial coating as it would prevent an aqueous bacterial suspension from forming droplets and allow a maximum surface area to volume ratio.

Table 3.1. Advancing and Receding Contact Angle and Contact Angle Hysteresis

Treatment	θ_A (degrees)	θ_R (degrees)	θ_H (degrees)
Native PP	104.5 ± 1.7^a	89.8 ± 0.9^a	14.4 ± 1.0^a
UV-O_3 PP	86.0 ± 2.0^b	51.1 ± 1.7^b	35.2 ± 2.0^b
Chitosan/Alginate	72.3 ± 1.7^c	43.8 ± 4.1^c	28.5 ± 3.0^c

*Values are means of three replicates. Treatments with the same letter within the same column are not significantly different ($P > 0.05$).

3.3.2 Surface Chemistry

ATR-FTIR spectra were measured to characterize surface chemistry of native PP and the modified PP coupons before and after 10 cycles of 200 ppm chlorine for 30 min. The spectra (**Figure 3.1**) show that the modified sample has additional bands, indicating the formation of new functional groups, compared to the native polypropylene. Native PP exhibited typical absorbances at 2960 – 2850 cm^{-1} and 1465 – 1440 cm^{-1} from $–CH_3$ and $–CH_2$ vibrations. Additionally, a characteristic band associated with the N-H bond of primary amines from the chitosan was exhibited between 1650 – 1590 cm^{-1}. The modified sample exhibited a broad band in the 3400 – 3200 cm^{-1} range, which corresponds to an O-H bond found in alginate. The characteristic band for the N-H bond of secondary amides was observed within the 1570 – 1515 cm^{-1} range and 1680-1630 cm^{-1} for C=O stretch. These bands indicate the formation of a covalent bond between amine of the chitosan and the carboxylic acid of the alginate during cross-linking (**Figure 2.2**). The band appears more prominent after 10 chlorination cycles, which could indicate a loss of weakly bound polyelectrolytes.

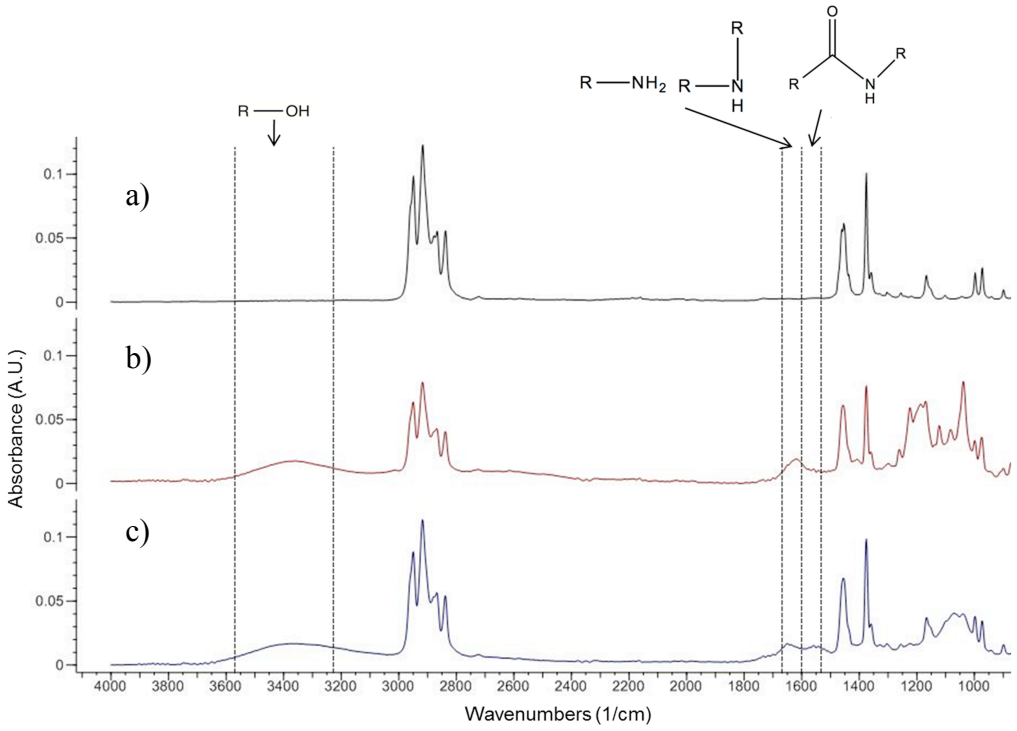

Figure 3.1. FTIR Spectra a) native polypropylene b) Chitosan/Alginate LbL c) Chitosan/Alginate LbL after 10 chlorination cycles of 200 ppm chlorine for 30 min per cycle.

The primary amine surface concentration was measured for native PP and modified sample using Acid Orange 7 dye (**Figure 3.2**). AO7 results indicated a significance difference ($P > 0.05$) between the polypropylene and the chitosan and alginate LbL treatment. Native PP showed no evidence of primary amines as expected. The chitosan and alginate LbL treatment had a relatively low amine content as a result of the chitosan not having as many amine groups due to its chemical structure and degree of deacetylation. The chitosan used had a deacetylation degree of ≥ 75%, as a higher number of amines are desirable for greater N-halamine stability and increased stability in a solution with an alkaline pH, such a sodium hypochlorite (Tan & Obendorf. 2007).

Further evidence supports that a higher deacetylation degree is associated with a greater level of bacteria growth inhibition of *Staphylococcus aureus* (Takahashi, et al. 2008).

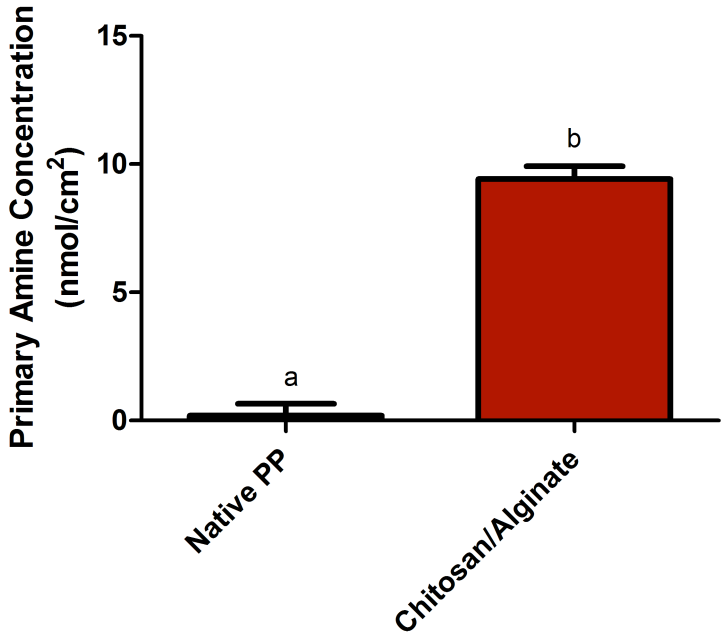

Figure 3.2. Primary amine content determined by Acid Orange 7 Dye Assay. Data shown are averages from two replicate assays (n=8). Treatments followed by the same letters are not significantly different ($P > 0.05$).

The stability of the coating was tested for 30 min in 200 ppm chlorine 10 times, or the equivalent to 300 sanitation cycles in a food processing plant (U.S. Food and Drug Administration. 2014). The chlorine content of the chitosan and alginate LbL sample experienced an initial decreased but stabilized off after 5 chlorination cycles and showed no significant change for the next 5 cycles ($P > 0.05$) (**Figure 3.3**). These results support the observations from ATR-FTIR as a small loss in the band corresponding to amides

was visible when comparing the spectra of modified samples before and after 10 chlorination cycles.

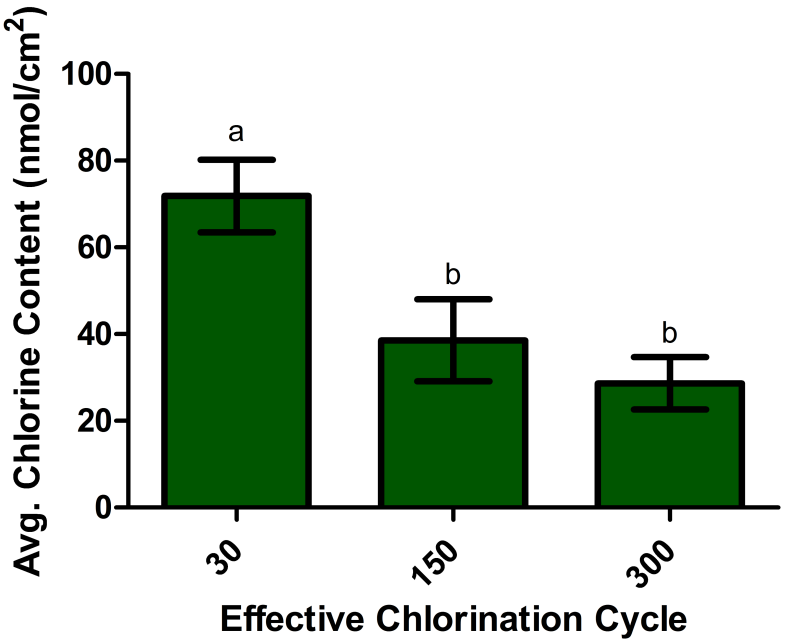

Figure 3.3 Chlorine holding capacity and regeneration ability over 10 chlorination cycles of 200 ppm chlorine for 30 min per cycle. Data shown are averages from two replicate assays (n=8).

3.3.3 Antimicrobial Activity

To determine the effectiveness of the chitosan and alginate coating, the modified and native polypropylene were chlorinated and exposed to aqueous bacterial suspensions for 2 h. An initial suspension of *L. monocytogenes* was plated prior to incubation to determine the starting concentration of bacteria, which had an average concentration of approximately 6 log CFU/mL. Plate counts from the antimicrobial activity tests revealed a > 5 logarithmic microbial reduction (> 99.999%) when compared to the native PP (**Figure 3.4**). The microbial load of the native polypropylene sample was greater than 6

log CFU/mL, indicating polypropylene has no biocidal effect and therefore the antimicrobial activity is derived from the chlorinated N-halamine coating alone. The chitosan alginate treatment obtained an inactivation that exceeded the limit of detection, which is less than 1 log CFU/mL.

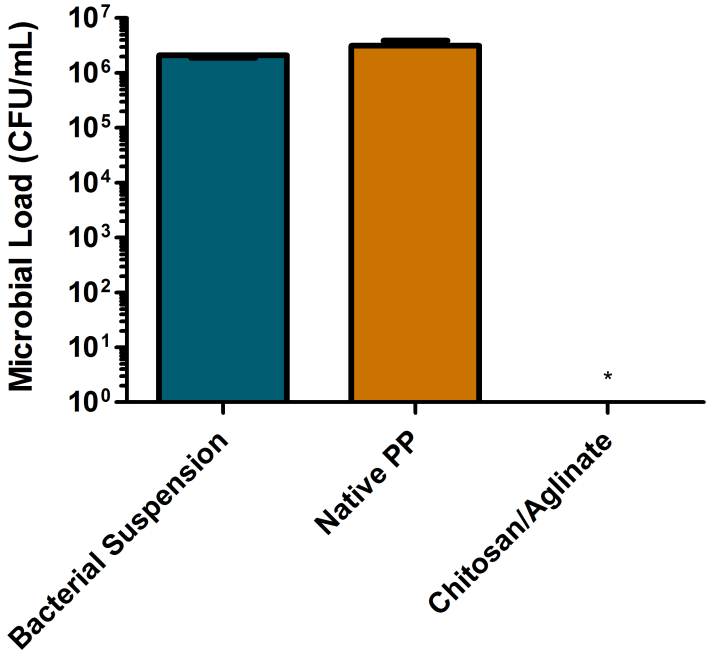

Figure 3.4. Antimicrobial activity of Chitosan/Alginate samples against *Listeria* after 2 hours of incubation with coupons at 32 °C (n = 8). *Treatment was below the limit of detection (< 1 log (CFU/mL)).

3.4 Conclusion

N-halamine antimicrobial coatings derived from natural sources are a promising method to inactivate *L. monocytogenes*. The chitosan and alginate coating was characterized through surface hydrophobicity; surface chemistry, using ATR-FTIR and a dye binding assay to quantify amines; and antimicrobial activity. The stability of the N-halamines in the coating was tested in 200 ppm chlorine over 300 equivalent sanitation

cycles in a food processing plant (U.S. Food and Drug Administration. 2014). An initial loss of N-halamines, and therefore chlorine content, was observed due to weakly bound polymers. However, the loss stabilized and remained constant for up to 300 minutes of exposure to sodium hypochlorite. Previous work has demonstrated that deposition of PEI and PAA by spray LbL assembly can enable > 5 logarithmic cycles (> 99.999%) reduction in the initial population of *L. monocytogenes.* In this study, preparation of N-halamine coating via spray LbL assembly with chitosan and alginate yielded a comparable reduction in the starting population of *L. monocytogenes*. Though there might be allergen or dietary concerns with chitosan sourced from crustaceans, it should be noted that chitosan could be sourced from fungi as well (Pochanavancih & Suntornsuk. 2002).

Spray N-halamine layer-by-layer deposition of chitosan and alginate is a rapid fabrication method using natural polymers with comparable antimicrobial effectiveness to coatings created from synthetic polymers.

CHAPTER 4

SUMMARY AND CONCLUSIONS

Microbial cross-contamination in food processing plants can lead to premature spoilage or the introduction of pathogenic bacteria into food. Current sanitation methods rely on cleaning with chemicals, such as sodium hypochlorite, at frequent intervals. However, cracks and crevices in a food processing plant can be challenging to clean, leading to reservoirs of bacteria. Antimicrobial coatings have promising potential to reduce microbial loads in between sanitation cycles. Coatings containing N-halamines, a refreshable antimicrobial moiety, have demonstrated effectiveness at inactivating bacteria. N-halamines can be formed via layer-by-layer deposition of polyelectrolytes with subsequent cross-linking.

Spray layer-by-layer deposition, was compared to a well-reported method, dip layer-by-layer fabrication using polyelectrolytes polyethylenimine and poly(acrylic acid). Spray LbL deposition was as effective as dip LbL deposition at inactivating *Listeria monocytogenes* with > 5 logarithmic reduction in the initial population. However, the N-halamines from both spray and dip LbL deposition methods were lost over the course of the equivalent of 300 sanitation cycles, 10 cycles in 200 ppm chlorine for 30 minutes, suggesting coating delamination.

Further work was completed using naturally sourced polyelectrolytes, chitosan and alginate, in spray layer-by-layer fabrication. A new method of introducing the cross-linker was used, resulting in increased stability in the N-halamine coating. After an initial decrease during the first 150 equivalent sanitation cycles the N-halamine content remained constant for the next 150 cycles.

In future work, cross-linking should be optimized to increase N-halamine stability after repeated exposure to chlorine sources. A few combinations of cross-linking methods (submerging in cross-linker solution, spraying cross-linkers in buffer solution, and spraying cross-linker in the polyelectrolyte solution) were tested in attempts to increase long-term stability. Though stability was increased in spray LbL samples of chitosan and alginate with cross-linkers sprayed onto the films, it still experienced an initial decrease in N-halamines. Alternative cross-linkers or application methods should be explored in order to mitigate this issue.

The effectiveness of the coating against wide range of microorganisms organism should be tested as only a Gram positive bacteria, *L. monocytogenes*, was challenged in the antimicrobial assay. The coupons of PP modified with chitosan and alginate layer-by-layers deposition should be tested against Gram negative and positive bacteria without chlorination to determine the base antimicrobial activity, if any, of the modified films without N-halamines.

Spray LbL deposition has the potential to be a rapid alternative to dip LbL fabrication as it can take less than 10% of the time as dip LbL fabrication and demonstrated comparable reduction in the starting population of *L. monocytogenes* of > 99.999%.

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