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Influence of Biomimetic Chelating Packaging on Natural Antimicrobial Efficacy

Paul Castrale

University of Massachusetts Amherst

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INFLUENCE OF BIOMIMETIC CHELATING PACKAGING ON NATURAL ANTIMICROBIAL EFFICACY

A Thesis Presented

By

PAUL A. CASTRALE

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

MASTER OF SCIENCE

September 2017

Food Science
INFLUENCE OF BIOMIMETIC CHELATING PACKAGING ON NATURAL ANTIMICROBIAL EFFICACY

A Thesis Presented

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PAUL A. CASTRALE

Approved as to style and content by:

______________________________________________
Lynne A. McLandsborough, Chair

______________________________________________
Eric A. Decker, Member

______________________________________________
Julie M. Goddard, Member

______________________________________________
Eric A. Decker, Department Head

Department of Food Science
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First and foremost, I must thanks to my advisors. Without their intuitive guidance, curiosity, and motivation I would not have been able to achieve what I have. Julie M. Goddard provided me with an opportunity to join her research group’s pursuit of biomaterial and biointerface discovery. As this area of research remains one of my primary interests, I am extremely thankful to have worked on this project. Julie has been a great teacher/mentor, and I’d like to thank her for guiding my understanding of how to be a researcher. Lynne A. McLandsborough graciously afforded me with space in her lab and council for my microbiology experiments. Lynne shared some of the very important pieces of microbiological knowledge that were invaluable to my experimental design. Maria G. Corradini has been a great source of enthusiasm and support. She kindly permitted me to continue my research in her lab space and become an “honorary member” of her lab. I’d like to thank Maria for remaining an extremely positive person, despite the equipment misfortunes that we’ve encountered. Eric A. Decker’s advice for my project has been greatly appreciated. I’d like to thank Yeonhwa Park, David Reckhow, Volodimyr Duzhko, and Julian Tyson for allowing me to use their equipment.

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ABSTRACT

INFLUENCE OF BIOMIMETIC CHELATING PACKAGING ON NATURAL ANTIMICROBIAL EFFICACY

SEPTEMBER 2017

PAUL A. CASTRALE

B.S., PURDUE UNIVERSITY

M.S., UNIVERSITY OF MASSACHUSETTS

Directed by: Professor Lynne A. McLandsborough

The iron chelating molecule, ethylenediaminetetraacetic acid (EDTA) is used in food applications for the preservation of oxidation prone ingredients. Research has suggested that EDTA is also capable of enhancing the antimicrobial effectiveness of various compounds including naturally-derived antimicrobials. With consumer demand for cleaner food labels, there remains an opportunity to introduce new chelating technology to replace synthetically-derived EDTA. Through photographting and chemical conversion, hydroxamic acid ligands were covalently bound to polypropylene films resulting in polypropylene- graft-poly(hydroxamic acid) (PP-g-PHA). The resulting films demonstrated an ability to chelate 64 nmol/cm² from an iron saturated environment or 163 nmol/cm² of magnesium and 139 nmol/cm² of calcium from bacterial growth media. A surface pKₐ of 8.97 suggested that film ligands should remain protonated under acidic and neutral pH conditions. When combined with lysozyme, PP-g-PHA films were able to reduce inhibitory concentration of lysozyme for Listeria monocytogenes by half. When tested against Bacillus cereus, Pseudomonas fluorescens, and E. coli O157:H7; PP-g-PHA films were unable to inhibit growth and showed little enhancement of lysozyme. EDTA
controls revealed that similar levels of soluble chelator were more effective than immobilized chelators. EDTA results also suggested that a chelating film with a higher affinity for iron (through coordination or ligand stability) may be able to control *B. cereus* growth. Both EDTA and PP-g-PHA caused *P. fluorescens* to produce siderophores (pyoerdines), suggesting that each treatment resulted in a low-iron growth environment. These findings suggest that surface bound chelating technology can affect the growth of *L. monocytogenes* and enhance the effectiveness of lysozyme. With improved surface chemistry (a higher binding constant with iron), this technology has the potential to influence the growth of other pathogens and spoilage microorganisms.
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LIST OF ABBREVIATIONS

Ca: Calcium
CFU: Colony Forming Units
Cu: Copper
DI: Deionized
EDTA: Ethylenediaminetetraacetic acid
Fe: Iron
Mg: Magnesium
Mn: Manganese
PBS: Phosphate Buffered Saline
$pK_{a}^{\text{bulk}}$: Acid Dissociation Constant of the Polymeric Surface
PP: Polypropylene
PP-g-PAA: Polypropylene-graft-poly(acrylic acid)
PP-g-PMA: Polypropylene-graft-Poly(methyl acrylate)
PP-g-PHA: Polypropylene-graft-Poly(hydroxamic acid)
TSA: Tryptic Soy Agar
TSB: Tryptic Soy Broth
CHAPTER 1

INVESTIGATING THE INFLUENCE OF BIOMIMETIC CHELATING PACKAGING ON NATURAL ANTIMICROBIAL EFFICACY

1.1 Introduction

The challenge of providing safe and nutritious food has increased in recent years due to consumer demand for the removal of ingredients deemed unhealthy. Such clean label and organic movements have been well documented by news agencies, consumer insights, and researchers alike.\(^1,2\) However, the reduction in food additive usage, particularly preservatives, impacts the overall quality, shelf life, and safety of some foods. The FAO has estimated that 95-115 kg of food is wasted per capita per year in North America and Europe.\(^3\) Additionally, it has been estimated that bacteria cause 3.6 million cases (39%) of foodborne illness each year in the United States.\(^4\) For these reasons, there is a need to discover new technological solutions to overcome the challenges of removing food additives.

Two primary causes of food spoilage are oxidation and microbial growth. Oxidative spoilage can be initiated by enzymes, irradiation, and the presence of transition metals. The principal ion associated with the generation of reactive oxygen species is iron. In order to reduce iron catalyzed oxidation, chelators such as ethylenediaminetetraacetic acid (EDTA) can be added to food and beverages to alter the redox potential of the metal ions.\(^5\) EDTA is approved for use in a number of food applications such as canned carbonated soft drinks (33 ppm), salad dressings (75 ppm), sauces (75 ppm), mayonnaise (75 ppm) and various canned products.\(^6,7\) However, due to its status as synthetic chemical, EDTA has been a recent target of consumers and retailers for removal from food formulations.
With the exception of fermented foods, a major focus of food preservation is preventing bacterial growth. Chemical additives, water activity, acidification, processing techniques, storage conditions, and competitive microorganisms are examples of technologies used to deter unintended microbial growth. The use of naturally derived antimicrobials is of increasing interest as they are considered clean label additives. For example, lysozyme is naturally available at high concentrations in chicken egg white and causes cell lysis through hydrolysis of peptidoglycan.\textsuperscript{8} However, natural antimicrobials are often economically infeasible for food production purposes. Thus, there is an opportunity for new technologies that can enhance the efficacy of natural antimicrobials like lysozyme.

EDTA and natural antimicrobials have been shown to exhibit a synergistic or additive relationship against the growth of bacteria in several studies.\textsuperscript{9–15} The additional lipopolysaccharide layer in the cell wall of gram negative bacteria provides protection from lysozyme catalyzed hydrolysis of peptidoglycan. Chelators are capable of LPS disruption via chelation of divalent cations (primarily magnesium and calcium) which may allow lytic agents to access the peptidoglycan and cell membrane layer.\textsuperscript{16–18}

In the search for EDTA replacement technology, researchers have identified siderophore functional groups (catecholate, hydroxamate, and $\alpha$-hydroxycarboxylate) as promising ligands for iron chelation.\textsuperscript{19,20} Siderophores are biological chelator molecules produced by many microorganisms in soluble-iron deficient environments which exhibit remarkable specificity for iron and high stability constants. The chemical synthesis of hydroxamic acid containing polymers capable of chelating iron has been researched for decades.\textsuperscript{21–23} However, polymer production has been chemically complex and resulted in
gel or free-floating polymers that are not feasible for a food application. Recent work by our group has produced chelating technology that is covalently bound to the surface of the common packaging material polypropylene (PP). An acrylic acid version of this technology demonstrated an ability to reduce the inhibitory level of lysozyme against *Listeria monocytogenes* by half. The overall goal of this work was to investigate the influence of hydroxamic acid (biomimetic) chelating films on various microorganisms relevant to food safety and spoilage. We hypothesize that hydroxamic acid should perform better than acrylic acid because it has a higher selectivity for iron and does not dissociate under acidic conditions (avoiding nonspecific fouling of cationic compounds).

The research goals of this work were to:

1. Synthesize chelating active packaging films by tethering hydroxamic acid chelating ligands onto polypropylene films. Characterize the surface chemistry and chelating performance of the materials.

2. Examine the influence of hydroxamic chelating materials on the growth of *E. coli* O157:H7, *Pseudomonas fluorescens*, *Bacillus cereus*, and *Listeria monocytogenes* with and without lysozyme.

In recognition of the importance of safe application of new technologies, the original objectives of this work were supplemented with research to characterize the migration level and effect of hydroxylamine from our films (see Appendix). Control variables based on the additional research were implemented for microbiological studies.
1.2 Materials & Methods

1.2.1 Materials

Polypropylene pellets (isostatic, Catalog # 130) were purchased from Scientific Polymer Products, Inc. (Ontario, NY). MES sodium salt was purchased from MP Biomedicals, LLC (Solon, OH). Hydroxylamine hydrochloride, methyl acrylate (99%), and EDTA disodium salt dihydrate were purchased from Acros Organics (Morris Plains, NJ). Ferric chloride hexahydrate, Pierce™ Bicinchoninic acid (BCA) protein assay kit, acetone, 2-propanol (isopropanol), methanol, hydrochloric acid (TraceMetal™ grade), nitric acid (TraceMetal™ grade), sodium phosphate dibasic heptahydrate, trichloroacetic acid, potassium chloride, sodium chloride, potassium phosphate monobasic, sodium bicarbonate, sodium carbonate, and sodium acetate trihydrate were purchased from Thermo Fisher Scientific (Waltham, MA). Imidazole, benzophenone, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p’-disulfonic acid monosodium salt hydrate (ferrozine, 97%), lysozyme from chicken egg white (≥ 98%, ≥ 40,000 units/mg), HEPES buffer, and methyl acrylate were purchased from Sigma Aldrich (St. Louis, MO). ICP-MS standard solutions (1,000 ppm) were purchased from Ricca Chemical Company (Arlington, TX). Bacto™ tryptic soy broth (TSB) and Difco™ tryptic soy agar (TSA) were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ). Ethyl alcohol was purchased from PHARMCO-AAPER (Brookfiled, CT).

1.2.2 Preparation of Polypropylene-graft-poly(hydroxamic acid)

PP-g-PHA films were prepared using an adaptation of the method established by Lin et al. (Figure 1). PP pellets were cleaned by two consecutive 10 minute sonication cycles in the following solvents: isopropanol, acetone, and deionized (DI) water. Clean PP
pellets were dried in a desiccator over anhydrous calcium sulfate for a minimum of 24 hours. PP films were prepared from dried pellets with a Carver Laboratory Press (Carver, Inc., Summit, NJ) compressed to deliver 8,500 pounds of pressure at 179°C. Film thickness was measured to be 0.47±0.04 mm (mean ± SD) by caliper (4 corners averaged for 141 samples). Pressed PP films were scored via scalpel on one side with a 4×4 cm square grid comprised of sixteen 1×1 cm squares. A 0.5 cm border around the scored grid was cut to produce 5×5 cm films. Cut and scored films were cleaned and dried in the same manner described for the PP pellets.

Cleaned PP films were subjected to 15 minutes of UV-Ozone treatment by a Jelight 42 (Jelight Co.; Irvine, CA) to increase wettability through hydrophilic group introduction. A grafting solution was prepared by dissolving benzophenone (5% w/v) in a methyl acrylate monomer. With a glass slide base, 0.35 mL of grafting solution was sandwiched under the UV/Ozone activated side of PP. Poly(methyl acrylate) (PMA) was photografted from PP by a 90 second UV curing process at 365 nm with a ~212 mW/cm² fluence (Dymax 5000 Zip Shutter Series; Torrington, CT). Grafted films were cut into 1×1 cm films and cleaned by overnight Soxhlet extraction in acetone (approximately 17 hours). Cleaned PP-g-PMA films were added to a reaction flask containing a solution of 5:1 methanol to water with 2.2 M sodium hydroxide and 1.3 M hydroxylamine hydrochloride. The hydroxylamine reaction flask was stirred at 70°C for 4 hours to convert poly(methyl acrylate) groups into poly(hydroxyamic acid).

PP-g-PHA films were rinsed in seven subsequent 5:1 methanol to water rinses. Rinse 1-3 and 5-7 were brief (~30 seconds). The fourth rinse was prolonged for 5 minutes and contained 0.2 M hydrochloric acid to fully protonate the hydroxamic acid ligands.
Following the seven rinses, PP-g-PHA films were washed with two 24 hour DI water cycles at 37°C (ratio of 6.75 mL/cm²). All PP-g-PHA films were dried with a Whatman HEPA-CAP™ filtered air gun and stored in a desiccator over anhydrous calcium sulfate for future use.

Figure 1: Changes in film chemistry during the production process. Yellow shading in PP-g-PHA washing diagram illustrates the theorized hydroxamic acid hydrolysis (not intended to suggest a ratio of ligands).
1.2.3 Iron Chelating Capacity

The ferrozine assay was used to quantify the ferric iron chelating capacity of PP-g-PHA with native PP as a negative control. The iron stock solution was prepared by dissolving ferric chloride hexahydrate (20 mM) in 0.1 M hydrochloric acid. Single 1×1 cm native PP or PP-g-PHA films were submerged in 5 mL of 50 mM sodium acetate and imidazole buffer (pH 5.0) containing 0.08 mM ferric iron. Blank samples of 0.08 mM ferric iron buffer without films served as an additional negative control to account for iron precipitation over the course of the study. Samples were shaken at 200 rpm for 24 hours in the dark conditions to allow for iron chelation.

Reducing reagent was prepared with 5% w/v hydroxylamine hydrochloride and 10% w/v trichloroacetic acid. Ferrozine reagent was made of 18 mM ferrozine in 50 mM HEPES buffer (pH 7.0). A standard curve (0.02-0.1 mM) was prepared from sodium acetate/imidazole dilutions of the 20 mM ferric iron stock. Iron buffer from each variable and standard curve sample (500 μL) was combined with 250 μL of the reducing and ferrozine reagents. Reaction mixtures were shaken for 1 hour at 21°C, aliquoted into clear 96-well plates, and analyzed for ferrozine-iron complex at 562 nm using a BioTek Synergy 2 plate reader (BioTek Instruments Inc.; Winooski, VT). A decrease in iron concentration compared to the blank samples was interpreted as film chelation. Reported values represent the average and standard deviation of two independent film preparations with 5 individual films per batch (n=10).

1.2.4 Surface Chemistry and Interfacial Dissociation Constant

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) was used to characterize the surface chemistry and determine the acid dissociation behavior of the
grafted surface (pK$_a^{\text{bulk}}$) of PP-g-PHA films. Buffered solutions of 0.1 M MES (pH ~3.0-7.5) and 0.1 M carbonate/bicarbonate (pH ~8.0-13) were prepared with 1 and 6 N sodium hydroxide or hydrochloric acid. Individual PP-g-PHA films (1×1 cm) were submerged in 2 mL of buffer for 1 hour at 21°C to allow ample time for proton exchange.

Buffered films were rinsed in ethanol, dried with nitrogen, and analyzed using an IRPrestige FTIR spectrometer with a diamond ATR crystal. ATR-FTIR spectra were obtained using the following parameters: absorbance, 32 scans per sample, Happ-Genzel model modification, and 4 cm$^{-1}$ resolution. Each pH condition combination was tested in quadruplicate and the spectra shown are representative of the four replicates.

Spectrum deconvolution of the 1900-1450 cm$^{-1}$ region was utilized for pK$_a^{\text{bulk}}$ analysis. The Fit Peaks (Pro) feature from OriginPro 2016 (OriginLab Corporation; Northampton, MA) was used to estimate the location and size of convoluted peaks. For each deconvolution, a user defined baseline based on two points at the bottom of the peaks (one on each side) was auto-subtracted the spectrum was rescaled. Estimated peak locations were user defined and the program fit curves based using a Gaussian distribution.

1.2.5 ICP-MS Analysis of Chelating Films in Growth Media

Inductively coupled plasma mass spectroscopy (ICP-MS) was used to determine the metal content of PP-g-PHA films after incubation in growth media. Single 1×1 cm PP-g-PHA or native PP films were submerged in 1 mL of 1:1 TSB to phosphate buffered saline (pH 7.3). Samples were incubated at 37°C with shaking (125 rpm) for 24 hours. Incubated films were rinsed 3 times with DI water from a wash bottle and dried with a filtered air gun. Film samples were held in a desiccator over anhydrous calcium sulfate
until acid digestion. Fresh PP and PP-g-PHA films from the same production batch were used as blank films for the ICP-MS analysis.

A Mars Xpress (CEM Corporation; Matthews, NC) microwave digestion unit was used to digest the polymeric films. Test variables consisted of: native PP, PP-g-PHA, native PP incubated in TSB, and PP-g-PHA incubated in TSB. Two 1×1 cm films were combined with 5 mL of nitric acid (TraceMetal™ grade) in 75 mL microwave digestion vessels (CEM Corporation; Matthews, NC). Standard curve samples were prepared with two 1×1 cm native PP films, 1 mL of pre-diluted ICP-MS standard solution, and 4 mL of nitric acid. Vessels were loaded into racks and heated in the Mars Xpress microwave via the following procedure: 20 minute ramp to 210°C, 10 minute hold at 210°C, and 10 minute cool down. Digested samples were transferred to 50 mL Falcon tubes, diluted to 25 mL, and stored at 3°C until ICP-MS analysis.

All acid digestion samples were diluted to 2% nitric acid with DI water prior to ICP-MS analysis. The final diluted concentration of each standard curve was 1-400 ppb. A PerkinElmer Elan DRC-e ICP-MS (Waltham, MA) was used to analyze the following elements: Fe, Mg, Ca, Cu, and Mn. Argon was used as the carrier gas. Three different film batches of each variable were analyzed in duplicate (n=6).

1.2.6 Effect of Chelating Films on Lysozyme

1.2.6.1 Bacteriological Media and Cultures

TSB and TSA were prepared as instructed by the manufacturer and autoclaved for sterility. Phosphate buffered saline (PBS; 1× strength) was adjusted to pH 7.3 with 1 M NaOH and sterilized by vacuum-assisted membrane filtration (0.22 µm).
Listeria monocytogenes Scott A and Pseudomonas fluorescens FSL W5-0203 cultures were provided by Dr. Martin Wiedmann (Cornell University; Ithaca, NY). Escherichia coli O157:H7 (ATCC 43895) was provided by Dr. Lynne A. McLandsborough (University of Massachusetts; Amherst, MA). Bacillus cereus (NRRL B-727, ATCC 13221) was provided by the Agricultural Research Service Culture Collection (Peoria, IL).

Bacteria were maintained in stock suspensions of 25% sterile glycerol and 0.5× strength TSB at -80 °C. Working cultures were cultivated from the bacterial stocks by suspending a loop of frozen stock culture in TSB. L. monocytogenes and E. coli were grown in 10 mL TSB tubes for 18-24 hours. All L. monocytogenes and E. coli experiments were completed at 37 °C with orbital shaking (125 rpm). P. fluorescens and B. cereus were cultivated in 100 mL Pyrex storage bottles with 20 mL TSB for 18-24 hours at 29°C with orbital shaking (300 rpm). Freshly grown cultures were streaked onto TSA plates and held at their respective temperatures for 24 hours. Plated working cultures were stored at 4 °C for up to two weeks.

For each experiment, one colony from a working culture plate was transferred into TSB and incubated for 16 hours. The TSB volume, container type, shaker speed, and temperature were consistent with the conditions used to grow the working cultures. Following growth, overnight suspensions were diluted 10000× in TSB to achieve an initial inoculum concentration of ~10⁵ CFU/mL.

1.2.6.2 24 Hour Effect of Chelating Films on Lysozyme

Lysozyme was added to PBS at a concentration of 8000 ppm. Lysozyme solvation was achieved by slow 3-D rotation mixing (Thermo Fisher Scientific; Waltham, MA) for 15 minutes at room temperature (~21°C). EDTA solutions were prepared by dissolving
0.128 g Ethylenediaminetetraacetic acid disodium salt dihydrate (0.100 g EDTA) in 50 mL of PBS (2,000 ppm EDTA). EDTA solutions were gently heated over flame (5-10 seconds total) with mixing to complete solvation. Hydroxylamine hydrochloride was dissolved into PBS at a concentration of 10000 ppm hydroxylamine. Additive solutions were filter sterilized (22 µm) and carefully diluted into sterile PBS. Additional additive dilutions were made in PBS.

Control variables were tested alongside PP-g-PHA test variables to validate the film’s chelating effect on lysozyme. Clean PP films were used as controls for all variables that did not include PP-g-PHA. EDTA controls compared the effectiveness of soluble and immobilized chelators. EDTA was incorporated at the same chelating concentration expected from four 1×1 cm PP-g-PHA films (75 ppm EDTA; Equation 1). Due to the chemistry of the PP-g-PHA films, the potential for hydroxylamine migration exists (see Appendix). Therefore, hydroxylamine controls were implemented to account for potential migration from four PP-g-PHA films over a 24-hour period (~5 ppm).

\[
\text{Eq 1: } 64 \frac{nmol \ Fe^3+}{cm^2} \times 4 \ cm^2 \times \frac{1 \ nmol \ EDTA}{1 \ nmol \ Fe^3+} \times \frac{1 \ mol \ EDTA}{10^9 \ nmol \ EDTA} \times \frac{292.24 \ g \ EDTA}{1 \ mol \ EDTA} \times \frac{10^6 \ \mu g \ EDTA}{1 \ g \ EDTA} \div 1 \ mL \ test \ volume = 74.81 \ \mu g \ EDTA/mL \ (ppm)
\]

Four PP-g-PHA or clean PP films were added to 13×100 cm tubes. Each tube was filled with 0.5 mL of ~10^5 CFU/mL inoculum in TSB and 0.5 mL of PBS. PBS fractions were comprised of 0.25 mL of PBS in combination with either 0.25 mL PBS, 0.25 mL lysozyme solution, 0.25 mL EDTA solution, or 0.25 mL hydroxylamine solution. Lysozyme test concentrations for L. monocytogenes were 0, 250, 500, 1000, and 2000 ppm. For all other bacteria, lysozyme was tested at 0 and 2000 ppm.
Sample tubes were incubated for 24 hours at ideal growth conditions for the test bacteria. After incubation, the samples were diluted in PBS and plated with TSA. The drop plate method was employed for *L. monocytogenes*, *E. coli*, and *P. fluorescens* tests with countable range of 15-150 CFU/50 µL. For enumeration purposes, *B. cereus* samples were pour plated with a countable range of 30-300 CFU/100 µL. The majority of bacteria were counted after 24 and 48 hours. *E. coli* was initially counted after 8-10 hours and then again at 24 hours. Reported values represent the mean and standard deviation of n=6 determinations (duplicate analysis of three independent film, media, and bacterial suspension preparations).

1.2.6.3 Mid-growth Effect of Chelating Films on Lysozyme

Variation in the growth behavior was investigated for *E. coli* O157:H7, *Pseudomonas fluorescens*, and *Bacillus cereus*. Time points within the respective bacterium’s logarithmic growth phase were sampled. *E. coli*, *B. cereus*, and *P. fluorescens* were tested at 3, 5, and 6.5 hours; respectively. Experimental preparation, growth conditions, and test variables were consistent with those used in the 24 hour microbiological studies. Reported values represent the mean and standard deviation of n=6 determinations (duplicate analysis of three independent film, media, and bacterial suspension preparations).

1.2.7 Pyoverdine from *Pseudomonas fluorescens*

Pyoverdines are a class of siderophores produced by pseudomonads which naturally contain a fluorescent dihydroxyquinoline group. Post 24 hour *Pseudomonas fluorescens* test samples were investigated for the presence of pyoverdine. Samples were centrifuged at 5000 ×g for 5 minutes to pelletize cellular growth. Supernatant from each
pelletized sample was added to black Corning 96-well plates. Plates were analyzed with the BioTek Synergy 2 using a 360/40 nm excitation filter and 460/40 nm emission filter. These filters were selected based on previous pyoverdine research.\textsuperscript{32,33} Incubated PP and PP-g-PHA samples without \textit{P. fluorescens} inoculum were considered blank samples and subtracted from the emission values of the test and control samples. Reported values represent the mean and standard deviation of \(n=6\) determinations (duplicate analysis of three independent film, media, and bacterial suspension preparations).

\textbf{1.2.8 Statistical Analysis}

One-way ANOVA (\(\alpha=0.05\)) and Tukey’s HSD multiple comparison tests (\(\alpha=0.05\)) were completed using GraphPad Prism 6 (GraphPad Software, Inc.; La Jolla, CA). Sigmoidal dose response model analysis for \(\text{pK}_a^\text{bulk}\) determination was also completed using GraphPad Prism 6.

\textbf{1.3 Results and Discussion}

\textbf{1.3.1 Iron Chelating Capacity}

The ferrozine assay was conducted to estimate the ferric iron chelating capacity of PP-g-PHA films at pH 5.0. After 24 hours incubation in 0.08 mM ferric iron, PP-g-PHA films chelated 64.03±9.92 nmol/cm\(^2\) ferric iron (Figure 2). PP-g-PHA iron chelating capacity was lower than previous work due to the additional film wash steps (see Appendix).\textsuperscript{24} Native PP films exhibited 6.14±10.15 nmol/cm\(^2\) of iron chelation. The impression of chelation by PP is likely due to iron deposition on the PP films.\textsuperscript{34}
1.3.2 Surface Chemistry and Interfacial Dissociation Constant

ATR-FTIR was used to determine the $pK_a^{\text{bulk}}$ of PP-g-PHA films based on previous work by our group.27 Peak deconvolution allowed for the elucidation of 1-2 peaks within the carbonyl stretch region of the FTIR spectrum (1760-1620 cm$^{-1}$). The peak occupying the 1610-1540 cm$^{-1}$ range is indicative of the “amide II” peak or carboxylate asymmetrical stretch and was not used in this analysis.35-37 The peak located in the middle of the deconvolution range was defined as the carbonyl stretch of the “amide I” (Figure 3).24 The third peak within the carbonyl region (1760-1620 cm$^{-1}$) represents the carbonyl stretch for carboxylic acid.

**Figure 2:** Iron chelation after 24 hours at pH 5.0 in 20 mM ferric chloride solution (n=10).

**Figure 3:** ATR-FTIR spectra for the characterization of PP-g-PHA carbonyl peak (gray) via deconvolution. Representative spectra of n=4 spectra per pH value. For each graph, x-axis: Wavenumbers (cm$^{-1}$) and y-axis: Absorbance
For pK$_{\text{a,bulk}}$ analysis, the “amide I” carbonyl peak location was plotted against the buffer pH and a sigmoidal curve function was employed to analyze the curve (Figure 4). The upper and lower limits for the curve were ~1669 and 1612 cm$^{-1}$. A stacked view of randomly selected pH values reveals the peak shift based of the “amide I” carbonyl peak (Figure 5). The inflection point of the sigmoidal curve signifies the pK$_{\text{a,bulk}}$ for PP-g-PHA and was determined to be 8.97, which falls within the reported pK$_{\text{a}}$ range of desferrioxamine B (8.35-9.71), a siderophore with hydroxamic acid groups. This pK$_{\text{a,bulk}}$ value is lower than what has been previously defined (9.65), an artifact of the carboxylic acid carbonyl peak skewing the “amide I” carbonyl peak towards 1700 cm$^{-1}$ under acidic conditions.

![Figure 4: Sigmoidal function curve for the “amide I” carbonyl peak shift based on deconvoluted spectra.](image-url)
**Figure 5:** ATR-FTIR the PP-g-PHA pH-dependent carbonyl peak shift. Representative spectra of n=4 spectra per pH value. Vertical lines represent the range of carbonyl shift defined by the sigmoidal curve (1669-1612 cm⁻¹). Spectra pH values randomly selected to provide an illustration of the peak shift.

### 1.3.3 ICP-MS Analysis of Chelating Films in Growth Media

The chelating performance of PP-g-PHA in TSB/PBS (growth media) was determined by ICP-MS. ICP-MS sample preparation limitations made the quantification of Fe, Mn, and Cu at appreciable concentrations impossible. Chelated compound data was calculated by subtracting the average analyte concentrations of blank PP or PP-g-PHA films from their TSB incubated counterparts. Presented calcium and magnesium data is based on isotopes Ca-44 and Mg-24 (Figure 6). PP-g-PHA films chelated a statistically higher concentration of Mg (163±10 nmol/cm²) and Ca (139±36 nmol/cm²) than PP control films (2.82±3 and 1.5±20 nmol/cm², respectively). Magnesium and calcium are important
for many vital functions in bacterial cells. \textsuperscript{39-41} Of particular importance to this study, magnesium and calcium chelation can decrease the integrity of gram negative bacterial cell walls. \textsuperscript{16,17}

\textbf{Figure 6:} ICP-MS results for PP-g-PHA and PP films following 24 hour incubation in TSB (37°C, n=6)

These ICP-MS results do not indicate that our films lack a high affinity for iron. It is hypothesized that the natural concentration of ions in TSB is the primary cause of these ion chelation results. Due to concerns with sample composition, TSB alone was not tested for metal content. Based on research by Damo \textit{et al.}, the concentration of trace elements in TSB is Mg > Ca > Fe > Mn > Cu with Mg and Ca at \(\sim 64 \times \) and \(\sim 23 \times \) the concentration of Fe. \textsuperscript{42} Furthermore, ICP-MS analysis of PP-g-PHA films following incubation in MRS broth, a growth media containing higher levels of Mn, revealed appreciable levels of Mn (110±6 nmol/cm\(^2\)), Ca (310±39 nmol/cm\(^2\)), and Mg (101±13 nmol/cm\(^2\)). Assuming the order of ion concentration is consistent with Damo \textit{et al.}'s work, our results reveal that PP-g-PHA films used in this analysis have a higher affinity for Ca compared to Mg. This result is consistent with a previous study on PP-g-PHA iron selectivity in the presence of competing ions. \textsuperscript{28}
1.3.4 Effect of Chelating Films on Lysozyme

Four microorganisms relevant to food quality and safety (*Pseudomonas fluorescens*, *E. coli*, *Listeria monocytogenes*, and *Bacillus cereus*) were evaluated to characterize the effect of the PP-g-PHA films on the antimicrobial performance of lysozyme. Hydroxylamine controls (PP + 5 ppm hydroxylamine) were statistically similar to PP controls for all 24 hour growth experiments and thereby not included in their respective figures.

1.3.4.1 *Listeria monocytogenes*

*Listeria monocytogenes* Scott A was susceptible to PP-g-PHA and EDTA treatments following 24 hours of incubation (Figure 7). PP-g-PHA films alone (0 ppm lysozyme) reduced the level of *L. monocytogenes* by 1.72 Log$_{10}$(CFU/mL) compared to PP controls. This result may be due to PP-g-PHA chelation of Mg or Ca cations (as evidenced by Figure 6). Comparatively, it is known that EDTA has a lower binding affinity for Mg and Ca, and a much higher affinity for Fe and Mn.$^{43}$ It is also possible that the bacteria reached full growth prior to the 24 hour time point and experienced a subsequent decline in population due to the presence of the chelating films.
Lysozyme at 2000 ppm was able to inhibit *L. monocytogenes* growth over 24 hours without a chelator (not shown). This growth inhibition level is consistent with previous research.\textsuperscript{26,44} However, it was much less effective than reported data from Branen and Davidson (inhibition at 250 ppm lysozyme).\textsuperscript{15} The discrepancy may be explained by variance in the lysozyme preparation. PP-g-PHA films exhibited a statistically significant reduction in viable cells compared to PP control films at each level of lysozyme from 0-1000 ppm.

The effectiveness of EDTA against *L. monocytogenes* Scott A was similar to previously reported results.\textsuperscript{15,26} EDTA at 75 ppm was unable to inhibit *L. monocytogenes* outgrowth after 24 hours. With the addition of 500 ppm lysozyme, no statistical difference was found between EDTA controls and the initial inoculum. There was a statistical difference (0.90 Log$_{10}$[CFU/mL]) between PP-g-PHA and the EDTA controls at 500 ppm of lysozyme. The difference increased by 2-3 Log$_{10}$(CFU/mL) at 250 ppm lysozyme (data...
only repeated once). The disparity between EDTA and PP-g-PHA results is considered evidence that soluble chelators (EDTA) are more effective than the immobilized chelators. *L. monocytogenes* does not produce siderophores.\(^{45,46}\) Therefore, its ability to multiply is greatly dependent upon iron availability. It is worth noting that the effects of chelators on *L. monocytogenes* may be reversed in environments containing heme or siderophores produced by other bacteria.\(^{45}\)

### 1.3.4.2 *Bacillus cereus*

Compared to other gram positive microorganisms, *Bacillus cereus* is more resistant to lysozyme due to the presence of N-nonsubstituted glucosamine in its peptidoglycan (i.e. N-acetylated substituents absent).\(^{47}\) Lysozyme’s inability to effect *B. cereus* on its own is evidenced by the PP plated results at 5 and 24 hours (Figure 8). While hydroxylamine did not affect the final cell concentration at 24 hours, it did inhibit growth at 5 hours. This growth inhibition may be a result of a prolonged lag phase, reduction of growth rate, or an initial reduction in microbial population. Previous research concluded that at “low” hydroxylamine concentrations (<3300 ppm), peroxide generation and DNA damage cause an inactivation of *Bacillus subtilis*.\(^{48}\) The same research also proposed that EDTA could reduce the effects of hydroxylamine on *B. subtilis*.\(^{48}\) Based on this work, it is expected that there was an initial reduction in viable bacteria upon hydroxylamine introduction. From a reduced initial inoculum level, hydroxylamine controls then grew to their cell concentration limit.

PP-g-PHA films were ineffective at controlling *B. cereus* outgrowth. In the presence of 2000 ppm lysozyme, PP-g-PHA films exhibited a slight reduction in viable cell counts (7.10±0.07 \(\log_{10}\) [CFU/mL]) compared to PP controls (7.31±0.16
Log₁₀(CFU/mL) at 5 hours of growth. However, the variance was not statistically significant. Aceto hydroxamic acid is similar to the intended ligand chemistry of PP-g-PHA and has a high ferric iron stability constant ([log β₁₁₀<sub>ferric</sub>]=28.29). However, with the randomized ligand organization, presence of carboxylic acids, and dependence on cation diffusion; it is expected that the PP-g-PHA films should not chelate iron as competitively as aceto hydroxamic acid.

![Graph](image)

**Figure 8:** *Bacillus cereus* plated results following incubation with PP-g-PHA, EDTA, and hydroxylamine (29°C, n=6). Letter designations indicate significance (p≤0.05). Lysozyme concentrations were independently analyzed for significance (i.e. One-way ANOVA) and separated based on Tukey’s HSD means comparison.

EDTA was an effective inhibitor of *B. cereus* growth. EDTA at 75 ppm alone resulted in no statistical difference compared to the initial inoculum level at 5 hours. The same control was higher than the initial inoculum concentration after 24 hours, but lower than PP controls (3.49 Log₁₀(CFU/mL) less). The combination of 2000 ppm lysozyme and EDTA had a significant effect on *B. cereus* compared to EDTA or lysozyme alone. The EDTA and lysozyme combination resulted in a 0.77 Log₁₀(CFU/mL) reduction of *B. cereus* compared to the initial inoculum at 5 hours. Following 24 hours of incubation, the combination of EDTA and lysozyme remained at parity with the initial inoculum level. These results suggest that EDTA at a concentration similar to the ferric iron chelating
capacity of PP-g-PHA is much more effective against *B. cereus*. Additionally, the experiment suggests that a chelating film with a higher specificity and capacity for iron could inhibit the growth of *B. cereus* under similar conditions.

Although EDTA is inhibitory at 75 ppm, the results show that *B. cereus* exhibits some growth between 6.5 and 24 hours alone (0.37 Log$_{10}$(CFU/mL) increase) and with lysozyme (0.46 Log$_{10}$(CFU/mL) increase). *Bacillus cereus* ATCC 14579 has shown the ability to produce the siderophores petrobactin (log$\beta_{110}^{\text{ferric}}$=~43) and bacillibactin ([log $\beta_{110}^{\text{ferric}}$]=47.6). These siderophores have higher unprotonated stability constants than EDTA ([log$\beta_{110}^{\text{ferric}}$]=25.1). Potential reasoning for the EDTA results include: *B. cereus* ATCC 13221 does not produce siderophores, does not produce high levels of siderophores under the test conditions, or is adversely effected by EDTA for reasons other than iron chelation. The effectiveness of EDTA in this study is in agreement with prior work that reported an inhibitory effect at <263, <248 and <100 ppm against *B. cereus*.

1.3.4.3 *Escherichia coli* O157:H7

For *E. coli* O157:H7, there was no appreciable difference between PP-g-PHA and PP control results at 24 hours (Figure 9). There was a 0.40 Log$_{10}$(CFU/mL) difference between PP-g-PHA and PP control results in the presence of 2000 ppm lysozyme at 3 hours. *E. coli* also showed limited susceptibility to hydroxylamine, which was not statistically differentiated from the PP-g-PHA results. Therefore, it is impossible to prove that hydroxylamine migration was not a factor in the PP-g-PHA results.
After 24 hours, EDTA at 75 ppm reduced *E. coli* O157:H7 viability by 0.28 Log$_{10}$(CFU/mL) alone and 0.68 Log$_{10}$(CFU/mL) in combination with 2000 ppm lysozyme. A similar reduction trend was observed for EDTA controls at 3 hours alone (0.50 Log$_{10}$(CFU/mL) less) and in combination with lysozyme (1.26 Log$_{10}$(CFU/mL)). These results suggest that *E. coli* growth is slowed in the presence of EDTA, and EDTA is more effective against *E. coli* when combined with lysozyme. Both of these findings are consistent with previously published works.\textsuperscript{15,55} *E. coli* produces a siderophore with one of the highest ferric iron stability constants, enterobactin ([log β$_{110}^{\text{ferric}}$]=49).\textsuperscript{56} Therefore, the eventual outgrowth experienced by EDTA controls was expected.

### 1.3.4.4 *Pseudomonas fluorescens*

*Pseudomonas fluorescens* growth was not inhibited by PP-g-PHA at 24 hours of incubation (Figure 10). The average cell growth at 6.5 hours for PP-g-PHA films was less than corresponding PP controls at 0 ppm lysozyme (0.11 Log$_{10}$(CFU/mL) reduction) and 2000 ppm lysozyme (0.37 Log$_{10}$(CFU/mL) reduction). However, the reductions were not great enough to declare statistical significance. Based on the 6.5 hour results, *P.*
fluorescens is susceptible to the bactericidal effects of hydroxylamine. Thus, any favorable data from PP-g-PHA samples is subject to speculation due to the potential for hydroxylamine migration.

**Figure 10:** *Pseudomonas fluorescens* plated results following incubation with PP-g-PHA, EDTA, and hydroxylamine (29°C, n=6). Letter designations indicate significance (p≤0.05). Lysozyme concentrations were independently analyzed for significance (i.e. One-way ANOVA) and separated based on Tukey’s HSD means comparison.

EDTA alone experienced a slight reduction in cell growth compared to PP controls at 24 hours (0.28 Log$_{10}$(CFU/mL)). But, in comparison to the initial inoculum, growth inhibition was minimal. The ability of *P. fluorescens* to grow in an iron limited environment is due its production of siderophores, primarily pyoverdines ([log $\beta_{110}$ferric]=30.8). At 6.5 hours, cell growth was not significantly different between EDTA alone and PP controls (0.37 Log$_{10}$(CFU/mL] difference). However, in combination with 2000 ppm of lysozyme, EDTA exhibited a significant 0.87 Log$_{10}$(CFU/mL) reduction compared to the PP and lysozyme control. A similar delay in *P. fluorescens* growth by ~50 ppm EDTA was previously reported. The 6.5 hour result for *P. fluorescens* is evidence that a combination of EDTA and lysozyme is more effective than either alone.
1.3.5 Pyoverdine Investigation

Centrifuged 24 hour *Pseudomonas fluorescens* samples from the microbiological assay were examined for relative pyoverdine content. Presented data represents variables that did not contain lysozyme. Sample data was normalized with incubated blank samples of PP or PP-g-PHA in sterile growth media. Results reveal an increase in the fluorescence of both PP-g-PHA and EDTA variables (Figure 11). This increase in fluorescence is expected to be the result of pyoverdine production. Despite the apparent difference in pyoverdine levels between samples exposed to PP-g-PHA and EDTA, the overall quantity of pyoverdine production is questionable due to the fact that pyoverdine fluorescence is quenched when complexed with iron\(^{33,59}\). Additionally, the production rate of pyoverdine is reduced as the organism reaches stationary phase\(^{60}\). These factors may account for the large difference in fluorescence between EDTA and PP-g-PHA samples.

![Figure 11: Apparent pyoverdine in solution following 24 hour growth of *P. fluorescens* (n=6). Letter designations indicate significance (p≤0.05). Means were separated based on One-way ANOVA and Tukey’s HSD means comparison.](image)

The PP and hydroxylamine control variables exhibit a negative fluorescence compared to PP blank samples. This reduction in fluorescent behavior may be the result of microbial metabolism of a fluorescent component native to TSB. Although their ideal
excitation and emission wavelengths are shorter than those used in this study, aromatic amino acids (tryptophan, tyrosine, and phenylalanine) may be causing the reduction in apparent fluorescence. Additionally, it is possible that an extracellular metabolite is quenching of the naturally occurring fluorescent compound.

1.4 Conclusion

PP-g-PHA films have demonstrated an ability to chelate 64 nmol/cm² of ferric iron from a 0.08 mM iron buffer at pH 5.0. ATR-FTIR analysis of the carbonyl peak shift revealed a pKₐ\textsubscript{bulk} of 8.97. Therefore, films should maintain minimal non-specific binding with charged polymers at growth media pH (7.3) due to acid protonation. ICP-MS results revealed PP-g-PHA films chelate 163 nmol/cm² of magnesium and 139 nmol/cm² of calcium from 1:1 TSB/PBS growth media over 24 hours. These results indicate that the PP-g-PHA films used in this study may have a higher stability constant with calcium than magnesium. Overall, PP-g-PHA characterization suggests that the films have the potential to effect microbiological growth through the chelation of cations.

PP-g-PHA films were able to reduce the amount of lysozyme necessary to inhibit *L. monocytogenes* growth over a 24 hour growth period by half. Although not statistically significant, PP-g-PHA in combination with 2000 ppm of lysozyme did provide a reduction in viable cells compared to PP lysozyme controls within the 6.5 hour *P. fluorescens* and 3 hour *E. coli O157:H7* experiments. After 24 hours of incubation, PP-g-PHA films with and without lysozyme were unable to control the growth of *P. fluorescens*, *E. coli O157:H7* or *B. cereus*. The ineffective nature of PP-g-PHA against these organisms may be due to the fact that the bacteria are capable of producing siderophores. This hypothesis is
supported by the fact that after 24 hours of growth in the presence of PP-g-PHA films, *P. fluorescens* growth media appeared to contain pyoverdines.

EDTA control treatments inhibited microbial growth better than PP-g-PHA in all microorganisms tested, suggesting that soluble chelators are more effective than immobilized chelators. In the majority of tests, the combination of lysozyme and EDTA was more effective than either treatment alone. *P. fluorescens* and *E. coli O157:H7* (gram negative bacteria) demonstrated a stronger resistance to EDTA than *B. cereus* and *L. monocytogenes* (gram positive bacteria). *B. cereus* was inhibited by the combination of 75 ppm EDTA and 2000 ppm at 24 hours. But, evidence of cell growth suggested that *B. cereus* was slowly producing siderophores to counteract the effect of EDTA. Assumed pyoverdine production was also evident in 24 hour EDTA treatments of *P. fluorescens*.

These results suggest that EDTA is able to increase the effectiveness of lysozyme against bacterial growth. Based on this and similar works, it is suggested that the chelator concentration and its affinity for iron are more important to inhibiting growth than the addition of lysozyme. The chelator’s affinity for iron is very important for effectiveness against microorganisms that produce siderophores. Developing polymerized chelating technology with a high affinity for iron will be challenging because stability constants are dependent upon coordination and ligand chemistry combined.
CHAPTER 2
HYDROXYLAMINE MIGRATION STUDIES

2.1 Introduction

The original synthesis procedure was modified with washing steps after determining that hydroxylamine was migrating from the films during BCA assay experiments. This chapter outlines the effect of washing PP-g-PHA on hydroxylamine migration and film chemistry. The films referred to as PP-g-PHA* did not undergo the two 24 hour DI washes following synthesis. PP-g-PHA films used in Chapter 1 are referred to by the same name in this Appendix.

In recognition of the importance of safe food contact materials, significant work was performed to characterize the migration of hydroxylamine from PP-g-PHA. Film wash studies were completed to reduce the extent of hydroxylamine migration, and proper controls were identified and implemented in microbiological work to account for the any residual hydroxylamine. Hydroxylamine is of microbiological concern due to its mutagenic interaction as a nucleophile and free radical promoter. Rosenkranz and Bendich demonstrated that the growth of *E. coli* C600 was inhibited by 33 ppm and slowed by 3.3 ppm of hydroxylamine.

2.2 Results and Discussion

2.2.1 Hydroxylamine Migration

In this work, it is hypothesized that hydroxylamine migration from PP-g-PHA* may originate from the residual hydroxylamine (from film synthesis) or acid hydrolysis of the chelating ligands. Indeed, the conversion of hydroxamic acid to carboxylic acid has been studied in a number of pH conditions and elevated temperatures. The proposed
mechanism for hydroxamic acid hydrolysis was concluded by Ghosh and is portrayed for the PP-g-PHA film chemistry in Figure 12.64

![Diagram of hydroxamic acid hydrolysis](image)

**Figure 12.** Proposed hydrolysis of hydroxamic acid groups in mildly acidic conditions.

Wash studies were conducted in order to reduce hydroxylamine migration from PP-g-PHA films. Hydroxylamine was quantified with the BCA assay. Hydroxylamine directly reduces cupric ions to cuprous ions which combine with BCA reagent to create a purple complex.66

To quantify hydroxylamine migration and establish effective wash conditions, four 1×1 cm films of PP-g-PHA*, PP-g-PHA, or native PP films were submerged in 1 mL of DI water. Water spiked with 20 ppm served as a positive control. All variables were incubated for 24 hours with shaking (125 rpm) at 37°C. Rinse water (0.1 mL) was combined with 2 mL of BCA reagent, and the hydroxylamine concentration was determined using the “in-tube BCA protocol” by conversion with a standard curve of 1-100 ppm. BCA assay tubes were incubated for 30 minutes at 37°C before absorbance
values were taken at 562 nm using a BioTek Synergy 2 plate reader. Reported values represent means and standard deviations of n=12 determinations (quadruplicate analysis of three independent film preparations).

Following 24 hours of incubation, hydroxylamine migration from PP-g-PHA and PP-g-PHA* films was 6.4±1.1 ppm and 54.0±6.5 ppm, respectively (Figure 13). Spiked hydroxylamine samples revealed no degradation at 20.53±0.98 ppm hydroxylamine. Native PP films produced negligible hydroxylamine generation (-0.02±0.27 ppm). Based on these results, hydroxylamine controls for microbiological studies were conducted at a concentration of 5 ppm.

![Figure 13: Hydroxylamine generation after 24 hour incubation of PP-g-PHA films (n=12)](image)

### 2.2.2 Surface Chemistry

To determine if hydroxamic acid hydrolysis was a cause of hydroxylamine migration, PP-g-PHA films were exposed to acidic conditions. Four 1×1 cm PP-g-PHA* films were individually incubated in 0.5 M HCl for 8 hours at 37°C. Native PP films were exposed to the same conditions and analyzed to ensure base film consistency. Acidified films were dried with a filtered air gun and stored in a desiccator. PP-g-PHA*, PP-g-PHA,
and acidified PP-g-PHA films from the same production batch were submerged in buffered solutions for 5 minutes at 21°C (4 films/20 mL). Buffers were comprised of 0.1 M MES (pH 4.01 & 5.96) or sodium carbonate/bicarbonate (pH 8.05 & 10.01). Prepared buffers were pH adjusted with hydrochloric acid or sodium hydroxide. Spectral peak analysis was completed using KnowItAll Academic Edition software (Bio-Rad Laboratories, Inc.; Hercules, CA).

In all pH buffers tested, PP-g-PHA and acidified PP-g-PHA films exhibited similar peak locations (Figure 14). PP-g-PHA* films maintained peak locations similar to previous ATR-FTIR analysis of PP-g-PHA.27 Peak locations pertaining to regions of interest are defined in Table 1. The apparent carbonyl stretch is represented by peaks within the 1760-1620 cm\(^{-1}\) range. Under acidic conditions, the carbonyl peak of PP-g-PHA and acidified PP-g-PHA films skewed towards 1681 cm\(^{-1}\). This carbonyl peak shift is indicative of a movement towards the typical carbonyl peak associated with acidified carboxylic acid dimers (1727-1705 cm\(^{-1}\)).24,27,35,36,67 An additional shoulder was observed on each film’s pH 4.01 spectra around 1730-1725 cm\(^{-1}\). This carbonyl peak is associated with either the carbonyl stretch of PP-g-PMA (1730 cm\(^{-1}\)) or carboxylic acid. PP-g-PHA* samples contain the characteristic “amide I” carbonyl peak at 1651 cm\(^{-1}\).24
Figure 14: ATR-FTIR spectra of acidified, PP-g-PHA, and PP-g-PHA* films at various pH values (n=4). Shading indicates region associated with a functional group. Magenta: 1760-1620 cm\(^{-1}\), Blue: 1610-1540 cm\(^{-1}\), Yellow: 1300-1200 cm\(^{-1}\)

Table 1: Apparent peak locations of acidified, PP-g-PHA, and PP-g-PHA* films at various pH values.

<table>
<thead>
<tr>
<th>Peak Identification</th>
<th>pH 4.01</th>
<th>pH 5.96</th>
<th>pH 8.05</th>
<th>pH 10.01</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>W</td>
<td>U</td>
<td>A</td>
</tr>
<tr>
<td>C=O</td>
<td></td>
<td></td>
<td></td>
<td>1681</td>
</tr>
<tr>
<td>C-N, N-O, or asym. CO(-)O</td>
<td>1543</td>
<td>1548</td>
<td>1548</td>
<td>1558</td>
</tr>
<tr>
<td>O-H, C-OH</td>
<td>1207</td>
<td>1213</td>
<td>-</td>
<td>1209</td>
</tr>
</tbody>
</table>

A: Acidified PP-g-PHA, W: PP-g-PHA U: PP-g-PHA*

Peaks associated with the 1610-1540 cm\(^{-1}\) region represent either carboxylate asymmetric stretching or “amide II” N-H bending and C-N vibration.\(^{35-37}\) Previous
research by our group suggests that the carboxylate stretch for polypropylene-
graft-poly(acrylic acid) is located at ~1566 cm\(^{-1}\), and the “amide II” stretch is located at ~1554 cm\(^{-1}\).\(^{27,28}\) Therefore, the slight difference in peak location at pH 8.05 and 10.01 may indicate a difference in the ratio of amide and carboxylate in the samples. Acidified PP-\(g\)-PHA and PP-\(g\)-PHA films have peaks nearer to the carboxylate stretching, while PP-\(g\)-PHA* films maintain a peak in close proximity to the “amide II” stretch.

The “fingerprint region” of 1300-1200 cm\(^{-1}\) is indicative of O-H bending and C-O stretching in carboxylic acid dimers.\(^{36}\) Broad peaks are evident in the acidified PP-\(g\)-PHA and PP-\(g\)-PHA samples from 1238-1207 cm\(^{-1}\). However, such peaks are absent in PP-\(g\)-PHA* films. It is worth noting that there is a shift in the peak locations for acidified PP-\(g\)-PHA and PP-\(g\)-PHA spectra between pH 8.01 and 10.01. The shift may be due to hydroxide interaction with the carbonyl group.

This spectral analysis data strongly supports our conclusion that hydroxamic acid hydrolysis is occurring during the film wash procedure. Based on peak intensities of the carbonyl and fingerprint regions (pH 4.01-8.05), it is suggested that the acidified PP-\(g\)-PHA samples may have experienced a higher degree of conversion than the PP-\(g\)-PHA samples. Acidified PP films showed no apparent spectral variance from native PP.

### 2.2.3 Ferrozine and pH of Wash Water

Ferrozine results varied as a result of PP-\(g\)-PHA washing. When compared to PP-\(g\)-PHA* films (92.64±15.80 nmol/cm\(^2\)), PP-\(g\)-PHA films exhibited a statistically significant decrease in chelating capacity. Hydroxamic acid ligands maintain higher ferric iron stability constants compared to carboxylic acids. Therefore, it is reasonable to suspect that the loss of chelating capacity in PP-\(g\)-PHA films may be a result of ligand alteration.
The pH of DI water rinsate from film wash cycles was examined for changes in pH. Triplicate analysis of two independent film washes (p=6) showed a significant difference between the change in pH of water (0.05±0.23) and PP-g-PHA water (2.70±0.78) over 24 hours in similar bottles. The increase in wash water pH, corresponding to proton absorption, substantiates the hydroxamic acid hydrolysis theory illustrated in Figure 12.

2.3 Conclusion

PP-g-PHA films are prone to hydroxamic acid hydrolysis. The primary concerns for this study are the migration of hydroxylamine, and a reduction in iron chelating specificity. As a result of this work, a wash procedure was implemented to reduce hydroxylamine migration, and a hydroxylamine control was identified for microbiological experiments (5 ppm).
CHAPTER 3
OUTLOOKS & PERSPECTIVES

3.1 Outlooks & Perspectives

Despite the relatively unfavorable results, this research does contribute to the body of work providing proof of principle for covalently bound chelating film technology. PP-g-PHA was able to influence the effectiveness of lysozyme against *Listeria monocytogenes* and appear to have contested *Pseudomonas fluorescens* for soluble iron. The challenges faced during these experiments allowed for enormous personal growth, an understanding of what it would take to achieve better results, and a precautionary warning against overlooking differences that may be significant.

The test conditions and film technology used in this research were not realistic for what would occur in a food system. The conditions used were ideal for microbial growth. Under more accurate conditions, the temperature, lack of aeration (if testing strict aerobes), and pre-exposure of the food matrix to chelating packaging may increase the effectiveness of the technology. However, the complex chemistry of the food matrix (lipids, complex carbohydrates, enzymes, iron containing proteins, additional cations, etc.) may provide a more challenging medium for chelating films than the growth media used in this study.

A few experiments that could be worthwhile for future technologies include:

- Conduct microbiological studies in growth media that has been subjected to chelating films for an extended period of time prior to inoculation. Perhaps the inoculum would be a concentrated cell suspension diluted into a buffer and added at a lower % volume ratio than this work. Pre-exposure conditions would better simulate the technology’s intended use.
- Conduct microbiological studies with a lower initial inoculum concentration. A lower starting inoculum would, in most cases, be a better approximation of contamination in the intended application of this technology.

- Conduct microbiological studies using sterilized foods/beverages (juices, sauces, dressings, etc.).

- Account for the surface area to volume ratio of model plastic containers (bottles).

- Test nisin at modified pHs (~5.0-6.0) for nisin solubility purposes.
LITERATURE CITED

(1) Paul Metz. Answering the Call for Clean Labels, 2016.


(3) FAO. *Global food losses and food waste - Extent, causes and prevention*.; Rome, 2011.


