INVESTIGATION OF BIODEGRADABLE POLY(ANHYDRIDE-ESTERS) HYDROLYSIS THROUGH IN VITRO MODELING AND KINETIC ANALYSES

by

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Written under the direction of Dr. Kathryn E. Uhrich

And approved by

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ABSTRACT OF THE THESIS

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Chemical incorporation of bioactives into polymers allows for their controlled and sustained delivery. Poly(anhydride-esters) (PAEs) are predominantly surface-erosing polymers, providing sustained release of bioactives. Compared to physical formulations, PAEs are capable of achieving higher bioactive loading (50 – 80 %) and have the versatility of being formulated into different geometries (discs, films), hence offering the potential to satisfy diverse administration routes in drug delivery. In this thesis, biodegradable polymers that contain salicylic acid and pinoresolvin as bioactives within PAEs are employed, and their hydrolysis investigated.

In one approach, a novel agar-based in vitro model mimicking the in vivo environment of the mandibular bone defect in diabetic animals was developed. Localized and systemic release profiles of salicylic acid from salicylic acid-based PAEs were evaluated. A sustained localized SA release was observed for 70 days. This study shows the agar-based system is a preferred model to mimic the in vivo mandibular bone defect
environment, and may be an appropriate method to mimic \textit{in vivo} pharmacokinetics of other drugs in related animal models.

In another approach, pinosylvin was chemically incorporated into a PAE backbone via melt-condensation polymerization, and fully characterized with respect to its physicochemical and thermal properties. \textit{In vitro} release studies in phosphate buffered saline (pH 7.4) demonstrated that pinosylvin-based polymers underwent slow hydrolytic degradation. Pseudo-first order kinetic experiments on butyric anhydride and 3-butylstilbene ester model compounds showed the anhydride underwent faster hydrolysis \([k = (30.6 \pm 2.24) \times 10^{-6} \text{ L mol}^{-1} \text{ s}^{-1}]\) than the ester \([k = (56.7 \pm 5.01) \times 10^{-7} \text{ L mol}^{-1} \text{ s}^{-1}]\). These results suggest that in solution, the anhydride linkages of pinosylvin PAEs are more labile and hydrolyzed first, followed by hydrolytic cleavage of the ester bonds to release pinosylvin. A disc diffusion antibacterial assay showed polymer release media exhibited similar bioactivity as free pinosylvin. \textit{In vitro} cytocompatibility studies demonstrated that the polymer was non-cytotoxic towards fibroblasts up to 0.5 \(\mu\text{g/mL}\). Based upon the results, pinosylvin-based polymers are slowly degradable biomaterials that provide antibacterial activity, and thus can serve as food additives into common food packaging materials for food preservation and food safety.
DEDICATION

This thesis is dedicated to my family: Wilfrid Bien-Aimé, Magaly Bien-Aimé, Sarahjine Wildlie Bien-Aimé, Jean-Richard Bien-Aimé, Dimitri Bien-Aimé, Thierry Joseph Bien-Aimé. Their support and encouragement have accompanied me throughout this journey.
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# TABLE OF CONTENTS

Abstract ii

Dedication iv

Acknowledgements v

Table of Contents vi

List of Tables ix

List of Schemes x

List of Figures xi

## CHAPTER 1: OVERVIEW

1

## CHAPTER 2: SUSTAINED, LOCALIZED SALICYLIC ACID DELIVERY VIA NOVEL *IN VITRO* MODEL MIMICKING *IN VIVO* ENVIRONMENT OF MANDIBULAR BONE DEFECT IN DIABETIC ANIMALS

2.1 Introduction 5

2.2 Materials and Methods

2.2.a SAPAE synthesis 8

2.2.b Sample preparation 8

2.2.c *In vitro* drug release study 9

2.3 Results and Discussion 10
2.4 Conclusion

CHAPTER 3: PINOSYLVIN-BASED POLYMERS: A NOVEL BIODEGRADABLE POLY(ANHYDRIDE-ESTER) FOR SUSTAINED RELEASE OF ANTIBACTERIAL PINOSYLVIN

3.1 Introduction 13

3.2 Materials and Methods

3.2.a Chemicals and reagents 15
3.2.b Structural characterization 15
3.2.c Molecular weight 16
3.2.d Thermal analysis 16
3.2.e Contact angle (CA) measurements 17
3.2.f 3,5-Dimethoxystilbene (2) synthesis 17
3.2.g Pinosylvan (3) synthesis 18
3.2.h Pinosylvan diacid (4) synthesis 19
3.2.i Pinosylvan monomer (5) synthesis 20
3.2.j Pinosylvan-based polymer (6) synthesis 20
3.2.k Pinosylvan monoacid (7) synthesis 22
3.2.l 3-Methoxystilbene (9) synthesis 22
3.2.m 3-Hydroxystilbene (10) synthesis 23
3.2.n 3-Butylstilbene ester (11) synthesis 24
3.2.o Relative rate of hydrolysis: kinetic experiments 25
3.2.p In vitro hydrolysis: bioactive release from pinosylvan-based polymers 26
3.2.q  *In vitro* hydrolysis: bioactive release from pinosylvin diacid 27

3.2.r  Antibacterial disc diffusion assay 27

3.2.s  *In Vitro* cytocompatibility studies 28

3.3  Results and Discussion

3.3.a  Physicochemical and thermal characterization 29

3.3.b  Relative rate of hydrolysis: kinetic experiments 31

3.3.c  *In vitro* hydrolysis: bioactive release from pinosylvin-based polymers 34

3.3.d  Antibacterial disc diffusion assay 35

3.3.e  *In vitro* cytocompatibility studies 37

3.4  Conclusion 38

REFERENCES 39

APPENDIX: RIGHTS AND PERMISSIONS 42
LIST OF TABLES

Table 1. Hydrolysis rate constants of model compounds that represent degradable linkages of pinosylvin PAE (6, Scheme 2), namely butyric anhydride (12) and 3-butylstilbene ester (11, Scheme 3)
LIST OF SCHEMES

**Scheme 1.** Hydrolysis of SAPAE, releasing SA and adipic acid as nontoxic by-product.

**Scheme 2.** Synthesis of pinosylvin-based PAE (6) from pinosylvin (3), an antimicrobial stilbene.

**Scheme 3.** Synthesis of 3-butylstilbene ester (11).
LIST OF FIGURES

Figure 1.  *In vivo* drug levels. (A) Traditional drug dosing with frequent administrations. (B) One dose of sustained release formulation within effective therapeutic window over a long period of time.

Figure 2.  (A) Bulk erosion where material is being lost throughout. (B) Surface erosion where erosion is limited to the surface of polymer.

Figure 3.  (A) Drugs covalently attached as pendant groups on a polymer backbone. (B) Drugs covalently attached within a polymer backbone as two bioactives per repeat unit. (C) Drug covalently attached within a polymer backbone as one bioactive per repeat unit.

Figure 4.  Schematic representation of the agar-based system mimicking the *in vivo* mandibular bone defect environment in diabetic animals.

Figure 5.  Schematic representation of the agar-based system mimicking the mandibular bone defect in *in vivo* settings. Samples were placed within plastic aperture and subsequently sealed in agar to mimic soft tissue surroundings. The overall agar system was incubated in PBS, and amounts of SA released into the PBS and within the plastic aperture were quantified to determine systemic and local SA release profiles, respectively.

Figure 6.  (Left) Concentrations of SA released within plastic aperture; (right) concentrations of SA release in PBS. (N = 3, mean ± SD).
Figure 7. (Left) Cumulative bulk SA release profile from agar study; (right) cumulative bulk SA release profile from previous work by Wada et al.

Figure 8. Butyric anhydride (12).

Figure 9. $^1$H NMR spectra highlighting key chemical shifts of 3,5-dimethoxystilbene (2, A); pinosylvn (3, B); pinosylvn diacid (4, C), and pinosylvn-based PAE (6, D).

Figure 10. FT-IR spectra of pinosylvn diacid (4) and pinosylvn PAE (6), comparing peaks of relevant functional groups.

Figure 11. Logarithmic plot showing kinetics of hydrolysis of butyric anhydride (12), monitored in PBS (pH 7.4) at $\lambda = 240 \text{ nm}$ and $37 \, ^\circ \text{C}$, at a cycle of 2 seconds. From the linear regression slope, $k_{\text{observed}}$ was extrapolated as the negative of the slope, then $k$ was calculated as $k = k_{\text{observed}} / [\text{H}_2\text{O}]$.

Figure 12. Rate constants for hydrolysis of 3-butylstilbene ester (11, Scheme 3) in 2.5%, 5%, and 10% 1,4-dioxane/PBS media. Hydrolysis rate constant of the ester in 0% 1,4-dioxane/PBS media was extrapolated from the linear regression line as the y-intercept.

Figure 13. In vitro hydrolytic degradation of pinosylvn PAE (6), releasing pinosylvn (3) over 40 days. $N = 3$ for each time point and data presented as mean +/- standard deviation. At the end of the study, a mass balance was performed (96 % mass accounted for) following analysis of bioactive in residual polymer.
Figure 14. Disc diffusion assay results for *E. coli* (A, C) and *S. aureus* (B, D) showing zones of inhibition ($Z_{IH}$) for free pinosylvin ($Z_{IH} = 17.5$ mm), extracted pinosylvin (3) ($Z_{IH} = 17.0$ mm), monoacid (7) ($Z_{IH} = 10.0$ mm) and diacid (4) ($Z_{IH} = 11.0$ mm) at 10 mg/mL in DMSO.

Figure 15. *In vitro* cytocompatibility of pinosylvin-based PAE (6) over a range of concentrations and times. Data is presented as mean ± standard deviation. $N = 6$ in each group. Statistical difference indicated by * ($p < 0.05$).
CHAPTER 1. OVERVIEW

Polymers are increasingly becoming significant biomaterials in the medical field as drug delivery vehicles.\(^1\)-\(^4\) Polymers as drug delivery carriers provide a sustained release of a therapeutic agent at a predictable rate over a long period of time.\(^4\)-\(^6\) Therefore, controlled delivery of a drug potentially increases patient compliance, and avoids the need for frequent administrations, which may result in drug overdose along with manifestation of its side effects (Fig. 1).\(^2\),\(^7\),\(^8\)

![Graph showing in vivo drug level](image)

**Figure 1:** *In vivo* drug level. (A) Traditional uncontrolled drug dosing with frequent administrations. (B) One dose of sustained drug release within effective therapeutic window over a long period of time.

Drugs are generally incorporated into polymers by physical or chemical methods. In physical incorporation, the drug is physically admixed with or encapsulated within the release-controlling polymer, then diffused from the polymer matrix over time. For
instance, antimicrobial carvacrol was admixed with poly(ethylene-co-vinylacetate) films\textsuperscript{9} and loaded in poly(lactic-co-glycolic acid) nanoparticles;\textsuperscript{10} however, these physical formulations suffer from low bioactive loading (<10 wt\%\textsuperscript{9}) and a burst release in which much of the antimicrobial (60\%) is released after only 3 hours.\textsuperscript{10}

On the other hand, chemical incorporation of bioactives into polymer matrices requires covalent bonding between polymer and drug, and that suitable functional groups are available. Although this approach may increase polymer water-solubility, offer higher drug loading, and sustained bioactive release, the majority of these polymeric pro-drugs, such as polymethacrylamide-based systems,\textsuperscript{11,12} ultimately do not degrade, and may constitute a drawback for applications in which implantable medical devices are involved. Biodegradable polymers have thus been explored as an alternative option.\textsuperscript{13-15} Such polymers, in addition to being inherently non-toxic, should degrade into safe components, and polyanhydrides constitute a good example in drug delivery.

Polyanhydrides are biocompatible biomaterials having hydrophobic backbone with hydrolytically labile anhydride linkages.\textsuperscript{14,16} The uniqueness of polyanhydrides lies in the fact these polymers are prepared from easily available low-cost resources, degrade hydrolytically \textit{in vitro} and \textit{in vivo} through chain scission process into their non-mutagenic and non-cytotoxic products, thus releasing bioactive agents at a controlled and predictable rate by surface erosion.\textsuperscript{14-16} Polyanhydrides are typically surface-eroding polymers, exhibiting rates of hydrolysis faster than rates of diffusion (Fig. 2, A).\textsuperscript{17-19} Erosion is therefore limited to the outer surface area of the polymer, allowing its core to remain intact, as opposed to bulk erosion where material is lost from the entire polymer
volume with no constant erosion velocity (Fig. 2, B).\textsuperscript{17-20} The valuable properties of polyanhydrides have gained the Food and Drug Administration’s approval of poly(sebacic acid-\textit{co}-1,3-bis(\textit{p}-carboxyphenoxy) propane) as a drug delivery carrier.\textsuperscript{16,21}

\textbf{Figure 2}: (A) Bulk erosion where material is being lost throughout. (B) Surface erosion where erosion is limited to the surface of polymer.

In synthesizing polyanhydrides, additional functional groups, such as esters, can be introduced in the polymer backbone to form bioactive-based poly(anhydride-esters) (PAEs), as developed by the Uhrich lab. PAEs are capable of covalent bioactive attachment, where bioactives are attached as pendant groups or within the polymer backbone via linker molecules (Fig. 3).\textsuperscript{22} In the latter case, bioactives can be incorporated either as two bioactives per repeat unit, or one per monomer (Fig. 3B and 3C).\textsuperscript{22} PAEs are predominantly surface-eroding polymers providing sustained release of bioactives with zero-order release kinetics.\textsuperscript{22,23} Furthermore, altering the chemistry of the polymeric backbone can modify degradation rates.\textsuperscript{22,24} Compared to physical formulations, PAEs are capable of achieving higher bioactive loading (50 – 80 %), and have the versatility of being formulated into different geometries (discs, hydrogels, microspheres, films), hence offering the potential to satisfy diverse administration routes in drug delivery.\textsuperscript{22,25}
In this thesis, the hydrolysis of well-established salicylic acid-based PAEs and newly synthesized pinosylvin-based PAEs is investigated. In the second chapter, an innovative model mimicking the *in vivo* environment of the mandibular bone defect in diabetic animals was developed to investigate release of salicylic acid from PAEs within the defect for more accurate local drug concentrations. The third chapter of this thesis addresses synthesis of novel pinosylvin-based polymers. Kinetics of hydrolysis of anhydride and ester bonds of model small molecules are investigated to better comprehend chemical degradation of pinosylvin-based PAEs in solution.
CHAPTER 2. SUSTAINED, LOCALIZED SALICYLIC ACID DELIVERY VIA NOVEL IN VITRO MODEL MIMICKING IN VIVO ENVIRONMENT OF MANDIBULAR BONE DEFECT IN DIABETIC ANIMALS


2.1 Introduction

Cytokines are proteins secreted by cells that have systemic anti- or pro-inflammatory effects on the body.\textsuperscript{26,27} Pro-inflammatory cytokines, such as prostaglandins and TNF-\(\alpha\), are produced by activated macrophages and contribute to inflammatory reactions.\textsuperscript{26,28} Studies in the literature have shown that elevated amounts of pro-inflammatory cytokines affect the activity and differentiation of osteoblasts and osteoclasts, and thus impair bone quality and healing, symptoms familiar to diabetic patients.\textsuperscript{29-32}

Diabetes is a metabolic disorder associated with hyperglycemia and/or hyperlipidemia due to insulin resistance or insulin deficiency, which is further enhanced by the increased release of pro-inflammatory cytokines.\textsuperscript{33,34} According to the American Diabetes Association, 9.3\% of the U.S. population in 2012 suffered from diabetes, more than 1 in 5 health care dollars goes to the care of people with diagnosed diabetes, and the calculated direct medical costs for the disease reaches $176 billion in the U.S. in 2012.\textsuperscript{35}
Bone impairment is a major complication of diabetes, and current treatment includes bone grafting and administration of growth factors such as bone morphogenic proteins (BMP).\textsuperscript{36-38} However, the former results in greater degree of variability in treatments and increased infection rates in patients,\textsuperscript{39} while the latter is exceedingly expensive with unresolved safety concerns and carcinogenic effects of BMPs \textit{in vivo}.\textsuperscript{40} Therefore, novel methods to address bone regeneration complications are needed, and salicylic acid offers an alternative to that end.

\textit{Salicylic acid (SA) is a well-established anti-inflammatory drug that reduces production of the above-mentioned pro-inflammatory cytokines impairing bone healing.}\textsuperscript{28} Previous work has demonstrated SA’s potency at significantly enhancing bone regeneration in diabetic rats where inflammation is expected, and at accelerating bone formation in normal rats, through local and continuous delivery of SA within a mandibular bone defect from a poly(anhydride-ester) (SAPAE).\textsuperscript{34} In this delivery system, SA was chemically incorporated into the polymer backbone and released via hydrolysis through surface erosion (Scheme 1).\textsuperscript{34} Duration of SA release from SAPAE can be altered to last from weeks to months, making it an ideal material to target chronic conditions.\textsuperscript{24} Consequently, SAPAE constitutes a promising method for treating diabetic bone healing compared to conventional bone grafting and growth factors. The hypothesized mechanism of action of SAPAE is that localized and sustained SA delivery prolongs anti-inflammatory effect observed in vivo, thus accelerating bone regeneration.
Scheme 1: Hydrolysis of SAPAE, releasing SA and adipic acid as nontoxic by-product.

To verify such a premise, a novel agar-based in vitro model mimicking the in vivo mandibular bone defect environment of diabetic animals, and equipped with an artificial bone defect site was developed. The overall agar system was placed in phosphate buffered saline (PBS, pH 7.4) to mimic physiological conditions. SA that was released into PBS and within the “defect” site was quantified to determine systemic (i.e., PBS) and local (i.e., agar) SA release profiles, respectively. SA concentrations in the bulk PBS showed negligible SA release for 2 days, followed by a rapid SA release at days 3-7, and then a slow and steady release for more than a month. Local SA concentrations at the artificial defect site also displayed a 3-phased profile: relatively low SA release for the first 2 days, followed by increased SA concentrations from days 3-6, then concluded with slow and sustained SA release. The localized and sustained SA release profile ascertained from this work correlates with the prolonged local anti-inflammatory effect observed in vivo by histological analysis (data not discussed here).41
2.2 Materials and Methods

2.2.a SAPAE synthesis

Synthesis of SAPAE was carried using the same methodology described in previous methods.24,25

2.2.b Sample preparation

Agar-based systems were designed to mimic the in vivo mandibular bone defect environment of diabetic animals for SAPAE degradation (Fig. 4). A plastic sheet (polystyrene, 0.55 mm thickness, LifeNet Health®, Virginia Beach, VA) with a 5 mm circular aperture was used to mimic the mandibular bone defect. A mixture of SAPAE, bone allograft (LifeNet Health®, Virginia Beach, VA), and light mineral oil (Sigma Aldrich, Milwaukee, WI) were placed in the plastic hole. Agar (0.015 g/mL, Sigma Aldrich, Milwaukee, WI) was used to surround and seal the plastic sheet to mimic the muscle and soft tissues surrounding the mandibular bone defect. Each agar-based system was assigned a specific date to be “sacrificed” (cut-open) for analysis.

![Figure 4: Schematic representation of the agar-based system mimicking the in vivo mandibular bone defect environment in diabetic animals.](image-url)
2.2.3 *In vitro* drug release study

Each system was submerged in 50 mL PBS (pH 7.4) (Sigma Aldrich, Milwaukee, WI) in a controlled environment incubator-shaker (New Brunswick Scientific Co., Edison, NJ) at 60 rpm at 37 °C to mimic physiological conditions (Fig. 5). At predetermined time intervals, agar systems were cut-open along the midline and SA from the plastic aperture was rinsed with 15 mL of PBS; this SA quantity should represent the local SA concentrations at the bone defect. The SA-containing release media was then analyzed by an Perkin Elmer Lambda XLS UV-Vis spectrophotometer (Waltham, MA) at $\lambda = 303$ nm, the maximum absorption wavelength for SA at which all other SAPAE degradation products minimally absorb.\(^\text{24}\) SA concentrations were calculated from a calibration curve of known standard solutions of SA. Before the cut-open time, 50 mL of the release media was removed every other day and replenished with 50 mL of fresh PBS to ensure sink conditions. The removed released media was also measured for SA concentrations determined by UV-Vis spectroscopy; this SA quantity represents systemic SA concentrations in animals. Study was performed in triplicate for each time interval.

**Figure 5:** Schematic representation of the agar-based system mimicking the mandibular bone defect in *in vivo* settings. Samples were placed within plastic aperture and subsequently sealed in agar to mimic soft tissue surroundings. The overall agar system was incubated in PBS, and amounts of SA released into the PBS and within the plastic aperture were quantified to determine systemic and local SA release profiles, respectively.
2.3 Results and Discussion

[Portions of the results and discussion are edited from Yu, W., Bien-Aime, S., Mattos, M., Alsadun, S., Wada, K., Rogado, S., Fiorellini, J. P.; Graves, D. T.; Uhrich, K. E. Biomaterials 2015, submitted.]

Published studies on SA-PAEs focus on systemic SA release, namely in the bulk PBS media. However, in the rat mandibular bone defect model, local SA concentration within defect is more therapeutically relevant, as it has more direct impact on bone regeneration than systemic SA concentrations. Therefore, this work presents a novel in vitro agar-based system that mimics the in vivo environment of the mandibular bone defect of diabetic animals, and more accurately models local SA concentrations. Furthermore, this innovative method avoids the need to euthanize an overly high number of animals.

Local SA concentrations in the plastic aperture displayed a 3-phase profile (Fig. 6, left). Before day 2, relatively low SA concentrations were monitored. The localized yet low SA concentrations at the early stage may allow for a low level of inflammation to take place after injury to initiate the necessary healing cascade. At days 3-6, a surge in local SA concentrations was observed. These higher levels of SA can suppress the overly high inflammation caused by pro-inflammatory cytokines that could hinder bone repair and formation, particularly in diabetic animals. From day 6 until end of study, a prolonged and slowed SA release was detected, which could ameliorate the chronic inflammation impairing bone healing in diabetic animals.
SA concentrations in the bulk PBS showed an initial lag period of 2 days, followed by rapid SA release at days 3-7, and concluded with a slow and steady release (Fig. 6, right). The localized and systemic SA release profiles were consistent in release rates and timing, with slightly more local SA (i.e., plastic aperture) concentrations observed for the first 2 days compared to bulk (i.e., systemic) SA concentrations. As more SA built up within the plastic aperture overtime, it dispersed to surrounding PBS by diffusion through agar. At the end of this study, about 60% (cumulative SA released at day 31) of the total SA incorporated in SAPAE was released into the surrounding media (Fig. 7). By data extrapolation, it is estimated that complete SA release will occur in 70 days. The bulk SA release profile is similar to the one shown in the previous in vivo study, where an initial lag period was observed and followed by sustained SA release (Fig. 7).\textsuperscript{34} However, the agar-based system provided a far longer SA release period (70 days for 100% SA release) compared to the previous study (16 days for 100% SA release) in which a mixture of SAPAE, bone allograft and mineral oil was directly placed in PBS without the shielding of agar.\textsuperscript{34} These findings show agar significantly hinders transport and delays SA diffusion. The 70 days time frame is comparable to the duration of the SAPAE treatments on diabetic rats from previous in vivo study, which lasted 12 weeks (84 days), and the effects of SAPAE on diabetic bone regeneration was observed throughout this period.\textsuperscript{34} Furthermore, the obtained data correspond to sustained local anti-inflammatory effect determined by histological analyses, indicating lower inflammation levels at the injury site on SAPAE-treated diabetic rats.\textsuperscript{41}
Figure 6: (Left) Concentrations of SA released within plastic aperture daily; (right) concentrations of SA released in PBS daily. (N = 3, mean ± SD).

Figure 7: (Left) Cumulative bulk SA release profile from agar study; (right) cumulative bulk SA release profile from previous work by Wada et al.\textsuperscript{34}

2.4 Conclusion

In this work, a novel agar-based \textit{in vitro} model mimicking the \textit{in vivo} mandibular bone defect environment of diabetic animals was developed. Localized and systemic release profiles of SA were evaluated. A sustained localized SA release was observed for 70 days. This data correlates with the prolonged local anti-inflammatory effect observed \textit{in vivo}, as demonstrated by histological analyses indicating lower inflammation levels at the injury site on SAPAE-treated diabetic rats.\textsuperscript{41} This study shows the agar-based system is a much better model to mimic the \textit{in vivo} environment, and may be an appropriate method to mimic \textit{in vivo} pharmacokinetics of other drugs in related animal models.
CHAPTER 3. PINOSYLVIN-BASED POLYMERS: A NOVEL BIODEGRADABLE POLY(ANHYDRIDE-ESTER) FOR SUSTAINED RELEASE OF ANTIBACTERIAL PINOSYLVIN

3.1 Introduction

Pinosylvin is an analog of resveratrol and a natural stilbene present in Pinus species.\textsuperscript{44,45} Owing to its intrinsic antimicrobial bioactivity, pinosylvin research has gained great interest. For instance, several studies have reported on pinosylvin antibacterial bioactivity against common foodborne pathogens such as Gram-positive (\textit{Staphylococcus aureus, Listeria monocytogenes}) and Gram-negative (\textit{Escherichia coli, Salmonella}) bacteria, even when the compound was physically admixed within food matrices of either vegetable or animal origin.\textsuperscript{46-48} The inherently valuable properties of pinosylvin have generated interest to incorporate the molecule into biomaterials to further its use, and such class of biomaterials is polymers as drug delivery carriers.

Polymers are increasingly becoming significant biomaterials in the medical field as drug delivery vehicles.\textsuperscript{1,2} Polymers as drug delivery carriers provide a sustained release of a therapeutic agent and avoid the need for frequent administrations.\textsuperscript{5,7} Since there is precedence in the literature for using polymers in extended drug delivery, the same concept is applicable to release bioactives such as pinosylvin in a sustained fashion as a natural food preservative. In an attempt to achieve extended release of stilbenes, researchers have physically encapsulated similar stilbenes into polymer matrices. For example, chitosan microspheres were loaded with resveratrol, yielding however
microspheres with low bioactive loadings (< 10%) and a burst release of the bioactive (over 60% within 30 min). As an alternative approach, chemical modification of resveratrol into a tri-acetyl ester has been attempted. However, complete in vivo hydrolysis of the ester-protected resveratrol was obtained within one hour. As resveratrol and pinosylvin are structurally similar, the physical and chemical modifications of pinosylvin, as described above, should result in the same outcomes as resveratrol. As a result, novel systems capable of achieving higher bioactive loading, and of providing extended release of pinosylvin as a food preservative are therefore of interest, and the chemical incorporation of pinosylvin into a PAE backbone offers an alternative to that end.

PAEs are predominantly surface-eroding polymers with minimal burst release, allowing for controlled and sustained release of bioactives. Additionally, PAEs can be prepared to achieve higher bioactive loadings (50 – 80 %), and formulated into different geometries, hence offering the potential to satisfy diverse applications.

In this work, the synthesis of pinosylvin-based polymers via melt-condensation polymerization, followed by characterization of its physicochemical and thermal properties, is presented. In vitro degradation studies in PBS (pH 7.4) were performed over 40 days and verified bioactive release resulting from polymer degradation. Pseudo-first order kinetic experiments were carried on butyric anhydride and 3-butylstilbene ester model compounds to understand chemical degradation of pinosylvin PAEs. The polymer cytocompatibility was assessed in vitro against fibroblast cells, and the release media confirmed antibacterial bioactivity via disc diffusion assay against Staphylococcus aureus
(S. aureus) and Escherichia coli (E. coli) bacteria.

3.2 Materials and Methods

3.2.a Chemicals and reagents

3,5-Dimethoxybenzyl bromide and 3-methoxybenzyl bromide were purchased from AK Scientific (Union City, CA). Concentrated hydrochloric acid (HCl), 1 N sodium hydroxide (NaOH), poly(vinylidene fluoride) (PVDF) and poly(tetrafluoroethylene) (PTFE) syringe filters, and Wheaton glass scintillation vials were purchased from Fisher Scientific (Fair Lawn, NJ). All other reagents, solvents, and fine chemicals were obtained from Aldrich (Milwaukee, WI) and used as received.

3.2.b Structural characterization

Proton ($^1$H) and carbon ($^{13}$C) nuclear magnetic resonance (NMR) spectra of all compounds were recorded on a Varian 500 MHz spectrophotometer. Samples (~5 mg/mL for $^1$H; ~50 mg/mL for $^{13}$C) were dissolved in either deuterated chloroform (CDCl$_3$) with tetramethylsilane as internal reference, or deuterated dimethyl sulfoxide (DMSO-$d_6$) as solvent and internal reference. Each spectrum was an average of 16 scans for $^1$H-NMR and 250 scans for $^{13}$C-NMR spectra. Fourier-Transform Infrared (FT-IR) absorbance spectra were measured on a Thermo Nicolet/Avatar 360 FT-IR spectrometer. Samples (1-3 wt%) were dissolved in dichloromethane (DCM) and solvent-cast onto sodium chloride (NaCl) plates. Each spectrum was an average of 32 scans.
3.2.c Molecular weight

Molecular weights (MWs) of the polymer precursors were determined from a Finnigan LCQ-DUO mass spectrometry (MS) running Xcalibur software equipped with an adjustable atmospheric pressure ionization electrospray source (API-ESI Ion Source). Samples were dissolved in methanol (10 µg/mL) and injected using a glass syringe. The pressure was kept at 0.8x10^{-5} Torr and the API temperature was maintained at 150 °C during the experiments. The weight-average molecular weight (M_W) of the polymer as well as polydispersity indices (PDI) were obtained by gel permeation chromatography (GPC) on a Waters liquid chromatography system consisting of a 515 HPLC pump, a 717 plus autosampler, and a 410 refractive index (RI) detector. Data analysis was then performed with Empower 2 software. Polymer samples were prepared for auto-injection by dissolving polymer in DCM (10 mg/mL) and filtering through 0.45 µm PTFE syringe filters. Samples were eluted through two PL gel columns 10^3 and 10^5 Å (Polymer Laboratories) in series at 25 °C, with DCM as eluent at a flow rate of 1 mL/min. M_Ws and PDIs were calculated relative to narrow M_W polystyrene standards (Polymer Source Inc., Dorval, Canada).

3.2.d Thermal analysis

Glass transition (T_g) and melting point (T_m) temperatures were determined via differential scanning calorimetry, which was performed using a Thermal Advantage (TA) Q200 instrument running on an IBM ThinkCentre computer equipped with TA Instrument Explorer software. Samples (4-8 mg) were heated under dry nitrogen gas to 150°C at a heating rate of 10°C/min and cooled to −50 °C at a rate of 10 °C/min with a two-cycle
minimum. TA Instruments Universal Analysis software 2000 (4.5A) was employed to analyze the data.

Decomposition temperature ($T_d$) of the polymer and precursors was obtained by thermal gravimetric analysis, which was performed on a Perkin-Elmer Pyris 1 system with TAC 7/DX instrument controller. Perkin-Elmer Pyris software running on a Dell Optiplex GX110 computer was used for automation and data collection and processing. Samples (5-10 mg) were heated under dry nitrogen gas from 25 °C to 500 °C at a heating rate of 10 °C/min, and $T_d$ values were measured at the onset of thermal decomposition represented by the beginning of a sharp slope on the thermograms.

### 3.2.e Contact angle (CA) measurements

Polymers (30 ± 5 mg, n = 3) were pressed into 8 mm diameter x 1 mm thick discs in an IR pellet die (International Crystal Laboratories, Garfield, NJ) with a bench-top hydraulic press (Carver Model 3925, Wabash, IN), with an applied pressure of 10,000 psi for 5 min at room temperature. Static contact angles (CAs) were measured by dropping deionized water onto pinosylvin polymer discs using a Ramé-Hart Standard Goniometer Model Number 250-00 (Mountain Lakes, NJ, USA), outfitted with a Dell Dimension 3000 computer with DROPimage Advanced software. Contact angles for each disc were measured in triplicate within 30 s of depositing water droplets onto the discs.

### 3.2.f 3,5-Dimethoxystilbene (2) synthesis

Synthesis of 3,5-dimethoxystilbene (2) was adapted from a procedure described elsewhere. Triethyl phosphite (P(OCH$_2$CH$_3$)$_3$) (1.6 eq) was added to 3,5-
dimethoxybenzyl bromide (1, 1 eq) in the presence of tetra-butyl ammonium iodine ((n-Bu)₄NI, 0.1 eq). The reaction was heated to 120 °C for 5 hours, and excess triethyl phosphite removed by heating under vacuum at 80 °C to generate diethyl[3,5-dimethoxybenzyl]phosphonate. The intermediate was then dissolved in 25 mL anhydrous tetrahydrofuran (THF) and reacted with benzaldehyde (1 eq) in the presence of sodium hydride (NaH, 2.5 eq). The mixture was allowed to stir at room temperature for 16-18 hours, and reaction progress was monitored by silica gel thin-layer chromatography (TLC) using 1:1 hexane to ethyl acetate (Hex: EA) mobile phase. Excess NaH was then quenched over chilled distilled water and acidified to pH 6 using concentrated HCl. The aqueous layer was extracted with ethyl acetate (EA, 4 × 50 mL) and washed with brine. The organic layer was dried over magnesium sulfate (MgSO₄), isolated via filtration, and the solvent was then removed in vacuo. The crude product was further purified via silica column chromatography using a 9:1 (Hex: EA) mobile phase to acquire 3,5-dimethoxystilbene (2) (Scheme 2).

Yield: 4.6 g (67%), light yellow crystal. ¹H-NMR (CDCl₃, 500 MHz): δ 7.51 (d, 2H, Ar-H); 7.36 (t, 2H, Ar-H); 7.30 (t, 1H, Ar-H); 7.06 (quartet, 2H, CH = CH, Δδ = 0.04, J = 16 Hz); 6.68 (s, 2H, Ar-H), 6.40 (s, 1H, Ar-H), 3.87 (s, 6H, OCH₃). ¹³C-NMR (CDCl₃, 500 MHz): δ 161.4, 139.8, 137.6, 129.6, 129.4, 129.2, 128.4, 127.2, 105.2, 100.6, 55.9 (2C). FT-IR (NaCl, cm⁻¹): 2927 (OCH₃), 1635 and 1592 (CH = CH). MS (m/z) = 240.1 [M]

3.2.g Pinosylvin (3) synthesis

Boron tribromide (BBr₃) (9 eq) in 10 mL anhydrous DCM was added to a solution of 2 dissolved in 20 mL anhydrous DCM at 0 °C (Scheme 2). The reaction was tallowed to
stir at 30 °C for 4 hours, was monitored by silica TLC using 1:1 (Hex: EA) mobile phase, then was quenched over chilled distilled water, and extracted with EA (4 × 80 mL). The organic layer was washed with brine, dried over MgSO₄, filtered, and the solvent was then removed *in vacuo* to obtain crude pinosylvin (3), which was further purified via silica column chromatography using a 3:1 (Hex: EA) mobile phase.

Yield: 1.4 g (35 %), light brown powder. $^1$H-NMR (DMSO-$_d_6$, 500 MHz): $\delta$ 9.21, (s, 2H, OH), 7.56 (d, 2H, Ar-H); 7.34 (t, 2H, Ar-H); 7.24 (t, 1H, Ar-H); 7.03 (quartet, 2H, CH=CH, $\Delta\delta = 0.04$, $J = 17$ Hz); 6.42 (s, 2H, Ar-H), 6.14 (s, 1H, Ar-H). $^{13}$C-NMR (DMSO-$_d_6$, 500 MHz): $\delta$ 161.4, 139.8, 137.6, 129.6, 129.4, 129.2, 128.4, 127.2, 105.2, 100.6. FT-IR (NaCl, cm$^{-1}$): 3300 (OH, phenol), 1632 and 1589 (CH = CH). MS ($m/z$) = 213.1 [M+1]

3.2.h  Pinosylvin diacid (4) synthesis

Synthesis of pinosylvin-based diacid (4) was adapted from a procedure described by Schmeltzer *et al* (*Scheme 2*). Pinosylvin (1 eq) was first dissolved in 30 mL anhydrous THF to which triethylamine (5 eq) was added. Glutaric anhydride (4 eq) was then added to the solution, and the reaction was stirred overnight at 60 °C and monitored by silica TLC using 1:1 (Hex: EA) mobile phase. The mixture was quenched over chilled distilled water, acidified to pH 2 with concentrated HCl, and the product was isolated via vacuum filtration to yield pinosylvin diacid (4).

Yield: 2.4 g (80 %), white powder. $^1$H-NMR (DMSO-$_d_6$, 500 MHz): $\delta$ 12.14 (s, 1H, OH, acid); 7.58 (d, 2H, Ar-H); 7.37 (d, 2H, Ar-H); 7.23-7.31 (m, 5H, Ar-H, CH=CH); 6.90 (s,
1H, Ar-H); 2.63 (t, 4H, CH₂); 2.35 (t, 4H, CH₂); 1.85 (quintet, 4H, CH₂). ¹³C-NMR (DMSO-d₆, 500 MHz): δ 179.5, 174.7, 171.9, 139.8, 137.6, 129.6, 129.4, 129.2, 128.4, 127.2, 117.9, 115.7, 34.8, 32.7, 19.4. FT-IR (NaCl, cm⁻¹): 2918 (OH, acid), 1759 (C=O, ester), 1709 (C=O, acid), 1610 and 1585 (CH=CH). MS (m/z) = 903.3 [(M × 2) + 23]. Tm = 151 °C. Td = 220 °C.

3.2.i Pinosylvin monomer (5) synthesis

Pinosylvin diacid (1 eq) was acetylated with excess amount of acetic anhydride (100 mL), and the mixture was stirred at room temperature overnight. Excess acetic anhydride was removed under reduced pressure to obtain pinosylvin monomer (5).

Yield: 2.7 g (94 %), dark brown paste. ¹H-NMR (DMSO-d₆, 500 MHz): δ 7.59 (d, 2H, Ar-H); 7.38-7.21 (m, 7H, Ar-H, CH=CH); 6.93 (s, 1H, Ar-H); 2.68 (s, 8H, CH₂); 2.22 (s, 6H, CH₃), 1.93 (s, 4H, CH₂). ¹³C-NMR (DMSO-d₆, 500 MHz): δ 171.7, 170.0, 167.7, 151.2, 140.1, 137.2, 131.2, 129.5, 129.3, 128.8, 127.4, 117.9, 115.7, 34.2, 32.9, 22.8, 19.7. FT-IR (NaCl, cm⁻¹): 1816-1750 (C=O, anhydride and ester), 1611 and 1588 (CH=CH). MS (m/z) = 547.0 [M + 23]. Tm = 155 °C. Td = 246 °C.

3.2.j Pinosylvin-based polymer (6) synthesis

Polymer 6 was synthesized according to previously published methods.⁵¹,⁵²,⁵⁵ Pinosylvin monomer (1 eq) was polymerized via melt-condensation polymerization at 170 °C under constant vacuum (< 2 mmHg) and constant stirring (100 rpm) using an overhead mechanical stirrer (Stirrer BDC1850, Caframo, Canada). Polymerization continued until the mixture solidified. The product was cooled at room temperature, dissolved in 10 mL
DCM, then precipitated over 100 mL chilled diethyl ether and isolated by vacuum filtration (Scheme 2).

Yield: 1.5 g (55 %), brown powder. $^1$H-NMR (DMSO-$d_6$, 500 MHz): $\delta$ 7.57 (d, 2H, Ar-H); 7.39-7.18 (m, 7H, Ar-H, CH=CH); 6.93 (s, 1H, Ar-H), 2.67 (s, 8H, CH$_2$); 1.91 (s, 4H, CH$_2$). $^{13}$C-NMR (DMSO-$d_6$, 500 MHz): $\delta$ 207.7, 171.7, 151.2, 140.1, 137.2, 131.2, 129.5, 128.8, 127.4, 117.9, 115.7, 34.2, 32.9, 19.7. FT-IR (NaCl, cm$^{-1}$): 1820-1750 (C=O, anhydride and ester), 1610 and 1587 (CH=CH). $M_w = 61,400$ Da; PDI = 1.3, $T_g = 50 \, ^{\circ}C$, $T_d = 190 \, ^{\circ}C$.

Scheme 2: Synthesis of pinosylvin-based PAE (6) from pinosylvin (3), an antimicrobial stilbene.
3.2.k Pinosylvin monoacid (7) synthesis

Pinosylvin-based monoacid (7) was adapted from a procedure described by Schmeltzer et al.\textsuperscript{55} Pinosylvin (1 eq) was first dissolved in 25 mL anhydrous THF to which triethylamine (0.7 eq) was added. Glutaric anhydride (0.7 eq) was added to the solution, and the reaction was stirred overnight at 60 °C and monitored by silica TLC using 1:1 (Hex: EA) mobile phase. The mixture was quenched over chilled distilled water, acidified to pH 2 with concentrated HCl, and the product was isolated via vacuum filtration to yield pinosylvin monoacid (7).

Yield: 0.90 g (81 %), brown paste. \textsuperscript{1}H-NMR (DMSO-\textit{d}_6, 500 MHz): δ 12.12 (s, 1H, OH, acid); 9.06 (s, 1H, OH, phenol), 7.57 (d, 2H, Ar-H); 7.38 (d, 2H, Ar-H); 7.23-7.31 (m, 5H, Ar-H, CH=CH); 6.90 (s, 1H, Ar-H); 2.65 (t, 2H, CH\textsubscript{2}); 2.32 (t, 2H, CH\textsubscript{2}); 1.87 (quintet, 2H, CH\textsubscript{2}). \textsuperscript{13}C-NMR (DMSO-\textit{d}_6, 500 MHz): δ 179.3, 174.5, 171.7, 139.9, 137.4, 129.5, 129.3, 129.6, 128.3, 127.1, 117.8, 115.6, 34.3, 32.5, 19.6. FT-IR (NaCl, cm\textsuperscript{-1}): 3300 (OH, phenol), 2916 (OH, acid), 1759 (C=O, ester), 1709 (C=O, acid), 1610 and 1585 (CH=CH). MS (\textit{m/z}) = 675.2 [(M × 2) + 23]. \textit{T}_\text{m} = 150 °C. \textit{T}_\text{d} = 215 °C.

3.2.l 3-Methoxystilbene (9) synthesis

Synthesis of 3-methoxystilbene (9) is adapted from a procedure described elsewhere.\textsuperscript{54} Triethyl phosphite (1.6 eq) was added to 3-methoxybenzyl bromide (8, 1 eq) in the presence of (n-Bu)\textsubscript{4}NI (0.06 eq) (Scheme 3). The reaction was heated to 120 °C for 5 hours, and excess triethyl phosphite removed by heating under vacuum at 80 °C to generate ethyl[3-methoxybenzyl]phosphonate. The intermediate was then dissolved in 25
mL anhydrous THF and reacted with benzaldehyde (1 eq) in the presence of NaH (2.5 eq). The mixture was allowed to stir at room temperature for 16-18 hours, and reaction progress was monitored by silica gel TLC, with a 1:1 (Hex: EA) mobile phase. Excess NaH was then quenched over chilled distilled water and acidified to pH 6 using concentrated HCl. The aqueous layer was extracted with EA (4 × 50 mL) and washed with brine. The organic layer was dried over magnesium sulfate (MgSO₄), isolated via filtration, and the solvent was then removed in vacuo. The crude product was further purified via silica column chromatography using a 9:1 (Hex: EA) mobile phase to acquire 3-methoxystilbene (9).

Yield: 5.0 g (66%), light yellow crystal. ¹H-NMR (CDCl₃, 500 MHz): δ 7.53 (d, 2H, Ar-H); 7.38 (t, 2H, Ar-H); 7.30 (t, 1H, Ar-H); 7.06 (m, 4H, CH = CH, Ar-H); 7.07 (s, 1H, Ar-H), 6.86 (d, 1H, Ar-H), 3.87 (s, 3H, OCH₃). ¹³C-NMR (DMSO-d₆, 500 MHz): δ 160.6, 139.5, 137.3, 129.5, 129.7, 129.6, 128.2, 127.3, 110.3, 111.2, 55.9 (1C). FT-IR (NaCl, cm⁻¹): 2927 (OCH₃), 1635 and 1592 (CH = CH). MS (m/z) = 443.2 [(M × 2) + 23].

3.2. m 3-Hydroxystilbene (10) synthesis

Synthesis of 3-hydroxystilbene (10) is adapted from a procedure described elsewhere.⁵⁴

Boron tribromide (BBr₃) (2 eq) in 10 mL manhydrous dichloromethane (DCM) was added to a solution of 9 dissolved in 20 mL anhydrous DCM at 0 °C (Scheme 3). The reaction was tallowed to stir at 30 °C for 4 hours, was monitored by silica gel TLC, with a 1:1 (Hex: EA) mobile phase, then was quenched over chilled distilled water, and extracted with DCM. The organic layer was washed with brine, dried over MgSO₄,
filtered, and the solvent was then removed \textit{in vacuo} to obtain crude 3-hydroxystilbene (10), which was further purified via silica column chromatography using a 3:1 (Hex: EA) mobile phase.

Yield: 1.8 g (36 %), light brown powder. $^1$H-NMR (DMSO-$d_6$, 500 MHz): δ 9.41, (s, 1H, OH), 7.59 (d, 2H, Ar-H); 7.36 (t, 2H, Ar-H); 7.27 (t, 1H, Ar-H); 7.03 (m, 4H, CH = CH, Ar-H); 6.96 (s, 1H, Ar-H), 6.69 (d, 1H, Ar-H). $^{13}$C-NMR (DMSO-$d_6$, 500 MHz): δ 160.6, 139.5, 137.3, 129.5, 129.7, 129.6, 128.2, 127.3, 110.3, 111.2. FT-IR (NaCl, cm$^{-1}$): 3300 (OH), 1630 and 1587 (CH = CH). MS ($m/z$) = 415.2 [(M × 2) + 23].

3.2. $n$ 3-Butylstilbene ester (11) synthesis

3-Hydroxystilbene (10) was first dissolved in anhydrous THF to which triethylamine (2 eq) was added (Scheme 3). Butyryl chloride (1.1 eq), dissolved in 10 mL anhydrous THF, was added to the solution over half-hour, and the reaction was stirred overnight at room temperature and monitored by silica gel TLC, with a 1:1 (Hex: EA) mobile phase. The solvent was removed \textit{in vacuo} followed by dissolution of the product in 2 mL of EA, which was washed with acidic water and brine. Mixture was then concentrated \textit{in vacuo} to yield 3-butylstilbene ester (11).

Yield: 0.16 g (68 %), brown powder. $^1$H-NMR (DMSO-$d_6$, 500 MHz): δ 7.59 (d, 2H, Ar-H); 7.47 (t, 1H, Ar-H); 7.38 (m, 4H, Ar-H); 7.27 (m, 3H, CH = CH, Ar-H); 7.02 (d, 1H, Ar-H); 2.57 (t, 2H, CH$_2$); 1.68 (sextet, 2H, CH$_2$); 0.99 (t, 3H, CH$_3$). $^{13}$C-NMR (DMSO-$d_6$, 500 MHz): δ 172.7, 150.6, 139.5, 137.3, 129.5, 129.7, 129.6, 128.2, 127.3, 121.3, 120.2, 37.5, 18.5, 13.6. FT-IR (NaCl, cm$^{-1}$): 1756 (C=O, ester), 1605 and 1582 (CH =
CH). MS ($m/z$) = 415.2 [(M × 2) + 23].

![Scheme 3: Synthesis of 3-butylstilbene ester (11).](image)

3.2.0 Relative rate of hydrolysis: kinetic experiments

*Studies performed with the assistance of Dr. Ralf Warmuth, Rutgers University, Department of Chemistry and Chemical Biology.*

![Figure 8: Butyric anhydride (12)](image)

Pseudo-first order kinetic experiments on butyric anhydride (12, Fig. 8) and 3-butylstilbene ester (11, Scheme 3) were performed on a UV-Vis recording spectrophotometer (Model UV-2401 PC, Shimadzu) equipped with a water bath (Model 1130A, VWR scientific). Butyric anhydride (12) and 3-butylstilbene ester (11) were chosen as model compounds representing anhydride and ester groups of pinosylvin polymer in study. Rates of hydrolysis of butyric anhydride (12) solutions ($4 \times 10^{-1}$ M, $n = 3$) in PBS (pH 7.4) were monitored under constant stirring at $\lambda = 240$ nm and 37 °C, at a cycle of 2 seconds. Because 3-butylstilbene ester (11) is not soluble in aqueous media, the ester was first dissolved in anhydrous 1,4-dioxane, and subsequently
diluted in PBS (pH 7.4) buffer. Rates of hydrolysis of 3-butylstilbene ester (11) solutions (6 × 10⁻³ M, n = 3) in 1,4-dioxane/PBS media (2.5%, 5%, 10%, 1,4-dioxane) were monitored under constant stirring at λ = 333 nm and 37 °C, at a cycle of 6 seconds. Hydrolysis rate constants of butyric anhydride (12) and 3-butylstilbene ester (11) were determined using logarithmic plots on excel, where \( k = k_{\text{observed}} / [H_2O] \) for a pseudo-first order reaction. In the case of 3-butylstilbene ester (11), rate constants for 0% 1,4-dioxane/PBS were extrapolated from the ones obtained for 2.5%, 5%, and 10% 1,4-dioxane/PBS media. Analysis of the kinetic data was done under the assumption that hydrolysis of the anhydride and ester model compounds was water-catalyzed (neutral pH region); i.e. not influenced by neither acid nor base catalysis mechanisms.

3.2.p \textit{In vitro} hydrolysis: bioactive release from pinosylvin-based polymers

Polymers 6 (60 ± 5 mg, n = 3) were pressed into 8 mm diameter x 1 mm thick discs in an IR pellet die (International Crystal Laboratories, Garfield, NJ) with a bench-top hydraulic press (Carver Model 3925, Wabash, IN), with an applied pressure of 10,000 psi for 5 min at room temperature. Polymer discs were incubated with PBS (20 mL, pH 7.4) in 20 mL Wheaton glass scintillation vials in a controlled environment incubator-shaker (New Brunswick Scientific Co., Edison, NJ) at 60 rpm and 37 °C. At predetermined time intervals, the media (20 mL) was removed and replaced with fresh PBS (20 mL) throughout the 40 days of study. Spent media were analyzed by high performance liquid chromatography (HPLC) using an XTerra RP18 3.5 μm 4.6 × 150 mm column (Waters, Milford, MA) on a Waters 2695 Separations Module equipped with a Waters 2487 Dual Absorbance Detector. All samples were filtered using 0.22 μm PVDF syringe filters and
subsequently injected (20 μL) using an autosampler. The mobile phase, modified from a published procedure, consisted of acetonitrile (40%) and 50 mM KH$_2$PO$_4$ in DI water with 1% formic acid at pH = 2.5 (60%). The mobile phase ran at 1.0 mL/min flow rate and 25 °C. Spent media absorbance was monitored at λ = 305 nm, and pinosylvin release was calculated from a calibration curve of known standard solutions of pinosylvin.

3.2.q **In vitro** hydrolysis: bioactive release from pinosylvin diacid

Diacid (4) was ground into powder using mortar and pestle to obtain particles of ~ 300 – 500 μm, as determined by standard testing sieves (Aldrich, Milwaukee, WI). Powdered diacid samples (5 mg) were in incubated in 20 mL of PBS (pH 7.4) in 20 mL Wheaton glass scintillation vials in a controlled environment incubator-shaker (New Brunswick Scientific Co., Edison, NJ) at 60 rpm and 37 °C. At predetermined time intervals, the media (1 mL) was removed and replaced with fresh PBS (1 mL). Spent media were analyzed by HPLC using the same methodology as described in above section (**In vitro** hydrolysis: bioactive release from pinosylvin-based polymers)

3.2.r **Antibacterial bioactivity**

*Studies performed with the assistance of Dr. Susan Skelly, Rutgers University, Department of Microbiology.*

Pinosylvin released from polymers was evaluated for antibacterial effectiveness against foodborne S. aureus and E. coli bacteria. First, the polymer was fully hydrolyzed using 1N NaOH, acidified to pH 2 with concentrated HCl, then extracted with EA (4 × 50 mL). The organic layer was dried over MgSO$_4$ and concentrated in vacuo to acquire extracted
pinosylvin. Free and extracted pinosylvin (3), as well as monoacid (7) and diacid (4) were each dissolved in DMSO to give a concentration of 10 mg/mL per sample. Free pinosylvin and DMSO were used as positive and negative controls respectively. Each sample was then tested against S. aureus and E. coli using a disc diffusion method by employing the following procedure. Muller-Hinton agar (Becton Dickinson, Sparks, MD) was poured into sterile Petri dishes (Fisher, Fair Lawn, NJ) to an even thickness of 4 mm. Each bacteria was suspended in nutrient broth (EMD Chemicals, Gibbstown, NJ) to give a bacterial count of approximately 10^8 colony forming units per mL. The agar plate was inoculated with bacteria broth culture using a sterile cotton swab (Fisher, Fair Lawn, NJ). Sterile paper discs (6 mm diameter, Becton Dickinson, Franklin Lakes, NJ) were impregnated with 20 µL of each solution (free pinosylvin, extracted pinosylvin, monoacid, diacid, and DMSO). Discs were then placed onto agar plates containing bacteria, and the plates were incubated at 37 °C for 24 h, after which zones of inhibition were measured to the nearest millimeter with a ruler.

3.2.s In Vitro cytocompatibility studies

[Studies performed by Weiling Yu, Rutgers University, Department of Biomedical Engineering.]

In vitro cytocompatibility studies were performed by culturing 3T3 mouse fibroblasts in Dulbecco's Modified Eagle's Media (DMEM, 10% Fetal Bovine Serum, 1% penicillin/streptomycin) containing Polymer 6 with different concentrations. The polymers were first sterilized under ultraviolet (UV) at \( \lambda = 254 \text{ nm} \) for 900 s, dissolved in DMSO and further diluted in cell media to yield 3 µg/mL, 1.5 µg/mL, 1 µg/mL, 0.5 µg/mL,
0.1 µg/mL and 0.05 µg/mL solutions. These concentrations were chosen to elucidate appropriate polymer concentrations for in vivo applications. Cell media containing polymers were then added to a 96-well plate with 2000 cells/well, and incubated at 37 °C. DMSO (1%) in cell media was used as control. Cell viability was determined using CellTiter 96® Aqueous One Solution Cell Proliferation Assay. After 24 h, 48 h, and 72 h incubation of the cells with polymers, 20 µL of (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) reagent was added to each well and further incubated for 4 h at 37 °C. The absorbance was then recorded with a microplate reader (Coulter, Boulevard Brea, CA) at 492 nm.

3.3 Results and Discussion

3.3.a Physicochemical and thermal characterization

Pinosylvin was chemically incorporated in a PAE backbone, yielding 51 wt% loading as outlined in Scheme 2; the synthesis of pinosylvin was adapted from a methodology employed elsewhere. Briefly, 3,5-dimethoxybenzyl bromide (1) was heated with triethyl phosphite in the presence of (n-Bu)_4NI, then the intermediate reacted with benzaldehyde to yield 3,5-dimethoxystilbene (2) (Fig. 9, 2A), followed by demethylation to generate pinosylvin (3) (Fig. 9, 3B). The chemical composition of pinosylvin was confirmed by IR spectroscopy and MS. Ring-opening of glutaric anhydride linker molecules by pinosylvin to generate pinosylvin diacid (4) was confirmed by the presence of the ester (C=O) and carboxylic acid (C=O) by FTIR spectroscopy (Fig. 10), and the relevant chemical shifts in the ^1H-NMR spectra (Fig. 9, 4C).
Figure 9: $^1$H NMR spectra highlighting key chemical shifts of 3,5-dimethoxystilbene (2, A); pinosylvin (3, B); pinosylvin diacid (4, C), and pinosylvin-based PAE (6, D).

Pinosylvin monomer (5) was formed by acetylation of diacid (4) in excess acetic anhydride, as confirmed by NMR and FT-IR spectra spectroscopy. As pinosylvin monomer (5) had a moderate $T_m$ (155 °C) and a high $T_d$ (246 °C), melt-condensation polymerization was possible. Melt-condensation polymerization of activated monomer 5 at 170 °C produced polymer 6, as confirmed via HNMR (Fig. 9, 6D) and FT-IR spectroscopy, which shows the presence of anhydride carbonyls and ester bonds, the preservation of the double bonds, and the disappearance of terminal carboxylic acids (C=O) (Fig. 10). Polymer 6 has a $T_g$ value of 50 °C, exhibited no $T_m$, decomposed at
190 °C, and exhibited a CA value of 84°.

Figure 10: FT-IR spectra of pinosylvin diacid (4) and pinosylvin PAE (6), comparing peaks of relevant functional groups.

### 3.3. Relative rate of hydrolysis: kinetic experiments

Hydrolysis rates of two small molecules that represent the PAE structure, butyric anhydride (12) and 3-butylnylstilbene ester (11), were investigated to better comprehend hydrolytic degradation of pinosylvin PAEs. Hydrolysis rate constants of the model compounds are shown on Table 1 and determined using the formula \( k = \frac{k_{\text{observed}}}{[\text{H}_2\text{O}]} \) for butyric anhydride (12) (Fig. 11). For 3-butylnylstilbene ester (11), hydrolysis rate constants for 2.5%, 5%, and 10% 1,4-dioxane/PBS media were determined as described
for the anhydride compound (12). Hydrolysis rate constants of the ester (11) for 0% 1,4-dioxane/PBS were extrapolated from the values obtained for 2.5%, 5%, and 10% 1,4-dioxane/PBS media (Fig. 12). As the percentage of 1,4-dioxane increased, the rate constants decreased; this effect is likely due to 1,4-dioxane dispersing water molecules and preventing water from interacting via hydrogen bonding with charged species in the transition state. Therefore, the activation energy of the transition state increased relative to that of water alone, and rate of ester hydrolysis consequently slowed. This observation confirms the water-catalyzed assumption made earlier, because for acid- or base-catalyzed hydrolysis reactions, addition of 1,4-dioxane would increase the hydrolysis rate constants.58

At 37 °C, butyric anhydride (12) manifested a faster hydrolysis rate constant \[ k = (30.6 \pm 2.24) \times 10^{-6} \text{ L mol}^{-1} \text{ s}^{-1} \] than 3-butylstilbene ester (11) \[ k = (56.7 \pm 5.01) \times 10^{-7} \text{ L mol}^{-1} \text{ s}^{-1} \]. Numerous studies in the literature have investigated the hydrolysis of similar anhydrides and esters, but they were performed under either acid- or base-catalyzed conditions (low and high pH values), at room temperature or higher temperatures (>>37 °C).59-63 Therefore, because the hydrolysis conditions and parameters are extremely different, no direct comparisons can be made between the hydrolysis rate constants obtained in the literature to the ones from this study. Nonetheless, the experimental data obtained from this work correlate with the fact that the anhydride bonds, being more labile, are more susceptible to hydrolysis compared to ester bonds.18,64,65
Figure 11: Logarithmic plot showing kinetics of hydrolysis of butyric anhydride (12), monitored in PBS (pH 7.4) at λ = 240 nm and 37 °C, at a cycle of 2 seconds. From the linear regression slope, $k_{\text{observed}}$ was extrapolated as the negative of the slope, then $k$ was calculated as $k = k_{\text{observed}} / [\text{H}_2\text{O}]$.

Figure 12: Rate constants for hydrolysis of 3-butylnitilene ester (11, Scheme 3) in 2.5%, 5%, and 10% 1,4-dioxane/PBS media. Hydrolysis rate constant of the ester in 0% 1,4-dioxane/PBS media was extrapolated from the linear regression line as the y-intercept.
Table 1: Hydrolysis rate constants of model compounds that represent degradable linkages of pinosylvin PAE (6, Scheme 2), namely butyric anhydride (12) and 3-butylstilbene ester (11, Scheme 3)

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<tr>
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<th>butyric anhydride</th>
<th>3-butylstilbene ester</th>
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<tr>
<td>$k$ (L mol$^{-1}$ s$^{-1}$)</td>
<td>$(30.6 \pm 2.24) \times 10^6$</td>
<td>$(56.7 \pm 5.01) \times 10^7$</td>
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3.3.c *In vitro* hydrolysis: bioactive release from pinosylvin-based polymers

Bioactive release from polymer discs was monitored *in vitro* by HPLC on polymer discs at physiological conditions (37 °C, pH 7.4). Polymer degradation through hydrolysis of anhydride and ester bonds is an important factor for controlled release of the stilbene. To evaluate the hydrolysis of pinosylvin PAEs, pseudo-first order kinetic experiments were performed on butyric anhydride (12) and 3-butylstilbene ester (11) model compounds. Based on the kinetic analysis on these small molecules, butyric anhydride (12) underwent faster hydrolysis [$k = (30.6 \pm 2.24) \times 10^6$ L mol$^{-1}$ s$^{-1}$] than 3-butylstilbene ester (11) [$k = (56.7 \pm 5.01) \times 10^7$ L mol$^{-1}$ s$^{-1}$]). These findings suggest that the anhydride bonds of pinosylvin PAEs, being more hydrolytically labile, hydrolyze first to yield pinosylvin diacid (4) followed by hydrolysis of the ester bonds to generate pinosylvin (3).

As shown in (Fig. 13), a minor amount (~ 5 %) of pinosylvin is quickly released into the degradation media of polymer 6. After the first two days, polymer 6 exhibited near-zero order release kinetics of pinosylvin (3). The polymer is relatively slow-degrading, releasing approximately 10 % of pinosylvin (3) in 40 days. This slow-degrading behavior of 6 is likely due to its relatively high hydrophobicity (CA 84°), and
complete release of pinosylvin (3) is expected in 28 months, based on data extrapolation (Fig. 13). At the end of the study, a mass balance was performed (96% mass accounted for) following analysis of bioactive in residual polymer.

![Graph](image)

**Figure 13:** In vitro hydrolytic degradation of pinosylvin PAE (6), releasing pinosylvin (3) over 40 days. N = 3 for each time point and data presented as mean +/- standard deviation. At the end of the study, a mass balance was performed (96% mass accounted for) following analysis of bioactive in residual polymer.

3.3.3 Antibacterial disc diffusion assay

To confirm the benign effects of polymerization processes on pinosylvin’s antibacterial bioactivity, a disc diffusion assay was carried against Gram-positive *S. aureus* and Gram-negative *E. coli* foodborne bacteria. The concentration of each substance (free pinosylvin, extracted pinosylvin (3), monoacid (7) and diacid (4) in DMSO was greater than the minimum inhibitory concentration (MIC) for pinosylvin (250 µg/mL) to evaluate inhibition zones on the inoculated agar plates. From the data collected (Fig. 14), both free and extracted pinosylvin (3) diffused from the discs and exhibited similar zones of inhibition against both bacterial strains. Pinosylvin monoacid (7) and diacid (4) also prevented bacterial growth, with diacid (4) showing slightly larger
zoned of inhibition. This difference may be due to the greater aqueous solubility of 4 (2.24 mg/mL) compared to 7 (0.80 mg/mL), therefore allowing diacid (4) to diffuse more readily through the hydrophilic agar plate. Furthermore, release studies have shown that diacid (4) breaks down readily into monoacid (7) and subsequently pinosylvin (3) within the timeframe the disc diffusion assay was performed (100% pinosylvin release in 18 hours). This observation, coupled with diacid’s aqueous solubility and larger diffusion area, would explain the greater zone of inhibition for 4. Overall, this assay demonstrates that polymerization processes did not affect pinosylvin’s inherent antibacterial bioactivity against two of the most common foodborne pathogens.

Figure 14: Disc diffusion assay results for *E. coli* (A, C) and *S. aureus* (B, D) showing zones of inhibition ($Z_H$) for free pinosylvin ($Z_H = 17.5$ mm), extracted pinosylvin (3) ($Z_H = 17.0$ mm), monoacid (7) ($Z_H = 10.0$ mm) and diacid (4) ($Z_H = 11.0$ mm) at 10 mg/mL in DMSO.
3.3.e  *In vitro* cytocompatibility studies

[Studies performed by Weiling Yu, Rutgers University, Department of Biomedical Engineering.]

All polymers were cytocompatible at 0.5 µg/mL, 0.1 µg/mL and 0.05 µg/mL over 72 hours (*Fig. 15*); i.e. no significant difference in cell viability was found between the polymer groups and the media control. However, polymers at 3 µg/mL, 1.5 µg/mL, and 1 µg/mL were cytotoxic toward fibroblasts. Since pinosylvin’s antibacterial MIC against common Gram-positive and Gram-negative bacteria is 250 µg/mL,⁴⁶ pinosylvin polymers will be toxic at such high concentrations in vivo (500 µg/mL of polymer needed for 250 µg/mL therapeutic effect of pinosylvin). Therefore, pinosylvin polymers are recommended to be used for external applications, such as preservatives in food packaging materials.

*Figure 15*: *In vitro* cytocompatibility of pinosylvin-based PAE (6) over a range of concentrations and times. Data is presented as mean ± standard deviation. N = 6 in each group. Statistical difference indicated by * (p < 0.05).
3.4 Conclusion

Naturally derived pinosylvin possesses intrinsic antibacterial bioactivities against common foodborne pathogen strains (Listeria, Staphylococcus, Salmonella, E. coli), and sustained release of pinosylvin can prolong its valuable antibacterial effects and further its usage as a natural food preservative. Attempts in the literature for achieving extended release of pinosylvin and similar stilbenes by use of polymers as delivery vehicles have been unsuccessful.\textsuperscript{49,50} As an alternative, the chemical incorporation of pinosylvin into a PAE backbone for the extended release of the bioactive is investigated in this work. Physicochemical and thermal testing revealed successful synthesis while \textit{in vitro} hydrolytic degradation of the polymer confirmed sustained bioactive release of pinosylvin over 40 days. To understand the chemical degradation of pinosylvin-based PAEs, pseudo-first order kinetic experiments performed on butyric anhydride (12) and 3-butylstilbene ester (11) model compounds revealed faster hydrolysis for the anhydride \([k = (30.6 \pm 2.24) \times 10^{-6} \text{ L mol}^{-1} \text{ s}^{-1}]\) compared to the ester \([k = (56.7 \pm 5.01) \times 10^{-7} \text{ L mol}^{-1} \text{ s}^{-1}]\). Pinosylvin released from polymer retained its antibacterial biological activity as observed via a disc diffusion assay. \textit{In vitro} cytocompatibility studies demonstrated that polymer 6 is cytocompatible up to 0.5 µg/mL, which concentration falling well below pinosylvin’s MIC of 250 µg/mL. As a result, pinosylvin PAEs constitute a promising technology to be employed for external applications, such as food additives into common food packaging materials for food preservation and food safety.
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