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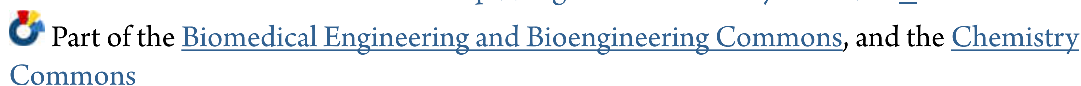
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**SYNTHESIS AND CHARACTERIZATION OF BIODEGRADABLE POLY(VINYL ESTERS) WITH HDAC INHIBITORY ACTIVITY**

by

**KYLE L. HORTON**

**THESIS**

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

**MASTER OF SCIENCE**

2013

MAJOR: BIOMEDICAL ENGINEERING

Approved by:

____________________________________________

Advisor Date

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**2013**

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# CHAPTER 1: INTRODUCTION

*HDAC inhibitors*

The acetylation and deacetylation of histones form an important and highly controlled regulatory mechanism in a cell's gene expression. Histone acetyltransferase (HAT) enzymes catalyze the addition of acetyl groups to the lysine residues of histones, negating their positive charge and permitting the DNA wound around them to loosen into a more relaxed and transcriptionally active state. Conversely, histone deacetylases (HDACs) catalyze the removal of acetyl groups from lysine residues and promote formation of a more compact chromatin. HDACs are capable of deacetylating a variety of other non-histone proteins as well; one review cites more than 50 proteins that have been identified (1). It has been suggested the ability of HDACs to deacetylate non-histone proteins gives them a variety of other functions and they should more accurately be called lysine deacetylases (1,2). Of the 18 known human HDACs and the four classes they are identified by, class I and class II HDACs contain a zinc-dependent active site that is competitively bound to by histone deacetylase inhibitors (HDACis). HDACis acting on zinc-dependent HDACs fall into four categories in order of decreasing potency: Hydroxamic acids (e.g. trichostatin A (TSA)), cyclic tetrapeptides, benzamides, and short chain carboxylic acids (e.g. valproic acid (VPA) and phenylbutyric acid (PBA)) (2,3) (Figure 1).

Since it was observed that HDAC activity is increased in cancer cells, HDAC inhibitors were first investigated as anti-cancer agents. HDAC inhibitors can induce the cell cycle regulator p21 in cancer cells and induce cell cycle arrest, thus inhibiting their proliferation (2). Two HDAC inhibitors, Vorinostat and Romidepsin, have been approved by the FDA for the treatment

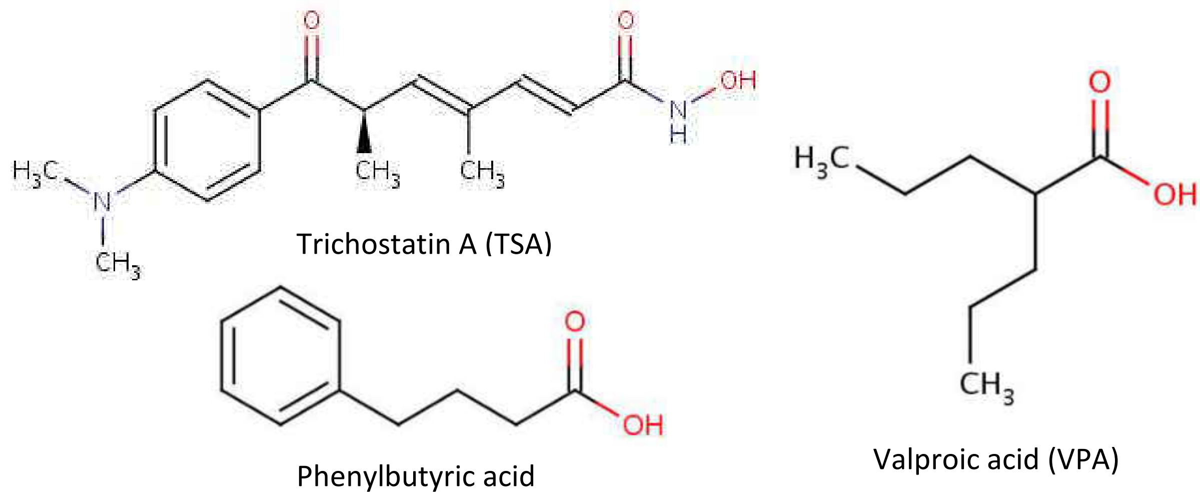



**Figure 1: A selection of HDAC inhibitors.**

of cutaneous T-cell lymphoma.

HDAC inhibitors also possess anti-inflammatory properties when used at significantly lower concentrations than those used in cancer treatment. In many autoimmune and inflammatory diseases such as rheumatoid arthritis (RA) and atherosclerosis there exists an improper activation of macrophages because of an overexpression of cytokines such as TNF-α, IL-6, IL-1β. The efficacy of anti-cytokine antibodies in reducing inflammation in these diseases demonstrates the central role cytokines play in their pathology (4,5). Whereas anti-cytokine antibodies must be delivered parenterally, HDAC inhibitors are orally active at low concentrations (4). Several studies have demonstrated a significant reduction in cytokine levels *in vitro* and in animal models when exposed to HDAC inhibitors (4). In one study, phenylbutyrate (2mM and 5mM) suppressed IL-6 and TNF-α production in human macrophages *in vitro* when stimulated with lipopolysaccharides (LPS) (5). Intact synovial biopsy explants from patients with RA stimulated by TNF-α in the presence of phenylbutyrate (1mM, 2mM and 5mM) displayed reduced production of IL-6, IL-8, IL-10, and a host of chemokines. No clear correlation

was found between macrophage histone acetylation and cytokine reduction. Considering the unintuitive observation that patients with RA and chronic obstructive pulmonary disease (COPD) display reduced HDAC activity at the disease site, it is likely that the targeting of non-histone proteins by HDACis plays a larger role in their anti-inflammatory activity.

*Induced Pluripotent Stem Cells*

In 2006, the team of Yamanaka et al. energized the field of stem cell research by successfully generating the first induced pluripotent stem cells (iPSCs). By forcing expression of four transcription factors (Oct4, Klf4, Sox2, and c-Myc) *via* retroviral vector in mouse embryonic fibroblasts (MEFs), a fully differentiated somatic cell type could be dedifferentiated into an embryonic stem (ES) cell-like state (6). The expression of these four genes is noted in embryonic stem cells for their ability to sustain a pluripotent state. The extent of equivalence between iPSCs and ESCs is under continued investigation, but there exist many commonalities including morphology, capability for unlimited self-renewal, expression of cell surface markers, demethylation of pluripotency gene promoters, and similarity in global DNA methylation. Like ESCs, iPSCs are capable of differentiating into multiple types of tissue. A common verification of pluripotency is to inject iPSCs in immunodeficient mice and observe the formation of teratomas containing tissue from all three germ layers. iPSCs were successfully generated from human fibroblasts using the same four transcription factors soon after (7).

The process for generation of iPSCs carries with it a number of drawbacks that have limited its potential for wide-scale adoption. The four-factor retroviral approach by Yamanaka et al. can only reprogram somatic cells at a very low rate (<1%) and the indiscriminate approach retroviruses take in inserting their genome poses a risk of mutation to the cell. Some

reprogramming factors (c-Myc in particular) are oncogenes and thus tumor formation is a major concern to be addressed. Alternate vectors have been investigated in an attempt to ameliorate these drawbacks: Adenoviruses (8), plasmids (9), and recombinant proteins (10) have all demonstrated success in producing iPSCs. However, each of these alternatives reduces the already low efficiency of reprogramming.

A promising route to the generation of iPSCs is the addition of small compounds to mimic and substitute for transcription factors or catalyze the process. In 2008, Melton et al. studied the effect of several small molecule compounds including the HDAC inhibitors suberoylanilide hydroxamic acid (SAHA), trichostatin A (TSA) and valproic acid (VPA) on four-factor transfection of MEFs (11). After one week of treatment, VPA (2 mM) appeared much more potent than any other compound and increased the percent of Oct4-expressing cells by >100-fold versus a non-treated four-factor control. When the expression of oncogene c-Myc was no longer forced, VPA increased efficiency by 50-fold versus a non-treated three-factor control. Importantly, three-factor transfection with VPA was more efficient than all four factors without VPA. VPA treatment did not affect the resemblance of iPSCs to ES cells in any way measured. Mouse embryos injected with VPA-treated iPSCs developed into healthy adult chimeras containing iPSC-differentiated cells from all three germ layers. These findings have generated interest in the transcription factor/small molecule compound combination approach to iPSCs.

*Layer-by-layer thin films*

Layer-by-layer deposition describes the process of assembling oppositely charged polyelectrolytes that are attracted by electrostatic forces on a surface. The deposition process

can be repeated to form multilayer nano-to-micro scale thin films. Polyelectrolytes for film formation can include both synthetic charged polymers and natural charged biomacromolecules such as DNA and proteins. Assembling a layer-by-layer film with biomacromolecules presents many advantages that make it an attractive approach to drug delivery and cell transfection (12). Because the layers are modular and contain discrete concentrations of biomacromolecules, the timing and order of delivery can be precisely controlled. More than one biomacromolecule can be delivered from the same film. The methods of film deposition permit their use on complex shapes at small scale, including particles and scaffolds. Layers can be fabricated from anionic plasmid DNA and cationic degradable polymers that form polyplexes, permitting the condensation and delivery of DNA into a cell.

*Hypothesis and specific aims*

HDAC inhibitors are known to have anti-inflammatory properties. HDAC inhibitors are used in combination with Oct4 to generate induced pluripotent stem cells. I hypothesize that polyesters based on simple aliphatic HDAC inhibitors like valproic acid (VPA) and phenylbutyric acid (PBA) can serve as alternatives to existing polyester biomaterials with improved anti-inflammatory properties and as scaffolds for generation of iPSCs when used in combination with layer-by-layer thin films delivering reprogramming transcription factors.

Specific aims of the study are as follows:

- Synthesize vinyl ester of valproic acid (VEVA) and vinyl ester of phenylbutyric acid (VEPA)
- Synthesize and characterize poly(VEVA) and poly(VEPA)

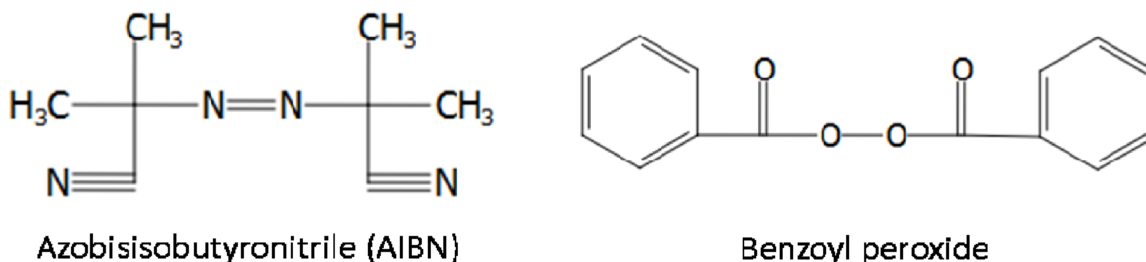
- Characterize hydrolysis of poly(VEVA) and poly(VEPA) and release of VPA and PBA
- Determine HDAC inhibition of poly(VEVA) and poly(VEPA)

## CHAPTER 2: SYNTHESIS AND CHARACTERIZATION OF HDAC INHIBITING POLYMERS

*Free radical vinyl polymerization*

A common and versatile method of synthesizing polymers is the chain growth of vinyl monomers by addition of free radical initiators. Many such polymers produced by free radical vinyl polymerization include poly(vinyl acetate), poly(methyl methacrylate), polyethylene, poly(vinyl chloride) and polystyrene. Two common initiators used in this process are benzoyl peroxide (BPO) and azobisisobutyronitrile (AIBN) (Figure 2). These symmetrical molecules have a functional group that can be decomposed by heat, separating them into fragment pairs containing free radicals (Figure 3). When the carbon double bond of a vinyl group of a monomer is introduced to an initiator fragment, the double bond is attacked by free radicals and opens up to accept a new electron pair with the initiator fragment. An unpaired electron is generated that can react with a new vinyl group and add to the growing polymer chain in sequence. Eventually, the process is terminated by recombination of two growing chain ends (coupling), or the abstraction of a hydrogen atom from one chain to another to form a terminal double bond (disproportionation). A variety of chain transfer agents act to stunt the growth of a polymer chain. Abstracting a hydrogen atom from solvent terminates chain propagation, necessitating careful selection of solvent type. Chain transfer to initiator terminates a chain

**Figure 2: Common free radical initiators.**



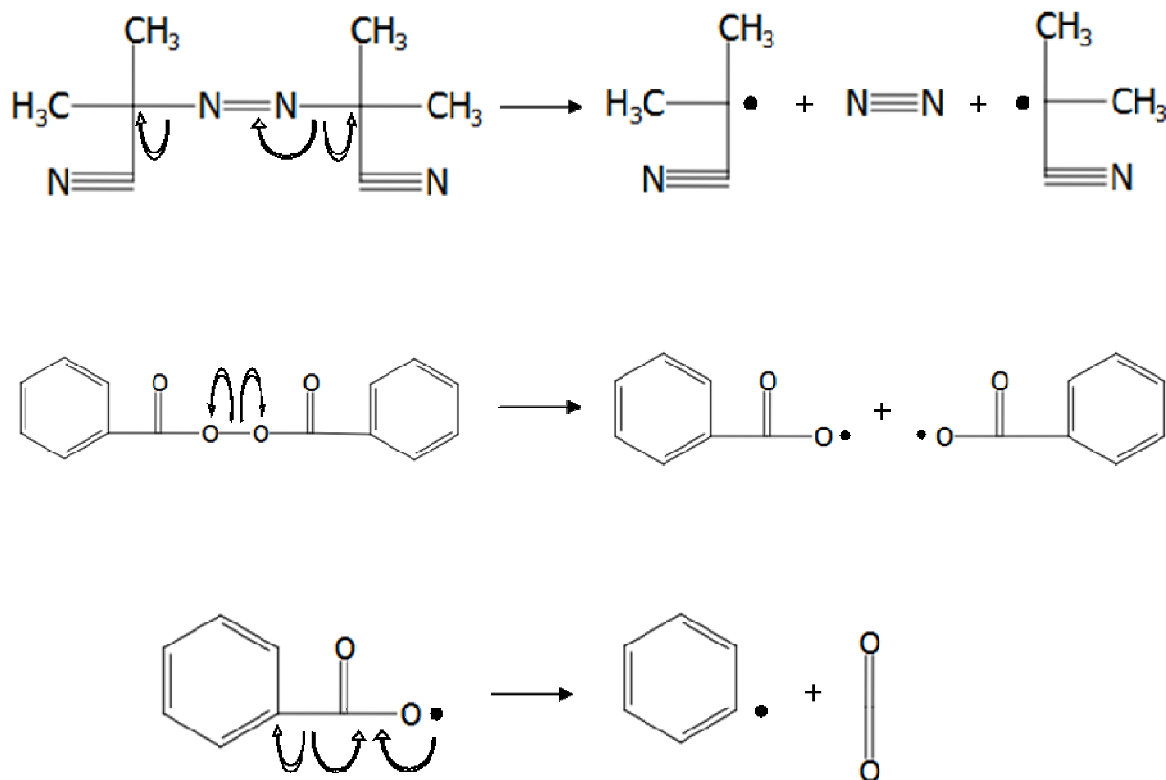



Figure 3: Thermal decomposition of AIBN and benzoyl peroxide.

while propagating a new one. If a hydrogen atom is abstracted from another chain, one chain continues propagating but the formed polymer becomes branched.

*Monomer synthesis*

In order to perform free radical vinyl polymerization, a vinyl group has to be present in the monomer. Vinyl esters are a commonly used intermediate in organic chemistry and several methods for their production exist in the literature and in industry. A common method of vinyl ester synthesis is the transvinylation of carboxylic acids with vinyl acetate catalyzed by transition metal complexes. Mercuric acetate is an efficient catalyst (13), but the toxicity of mercury makes it undesirable. Palladium is a well-established industrial catalyst (14) but delivers poor yield (15). Some ruthenium compounds are suitable catalysts, but they demand elevated reaction temperatures (16). Nakagawa et al. demonstrated the effectiveness of an

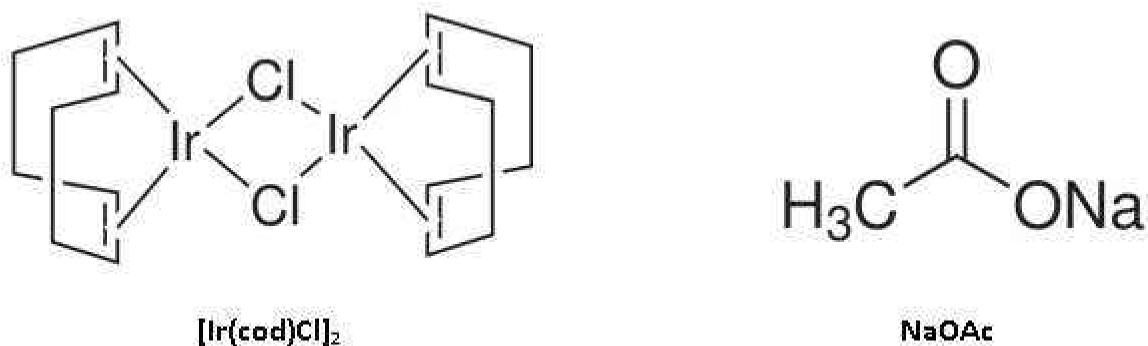
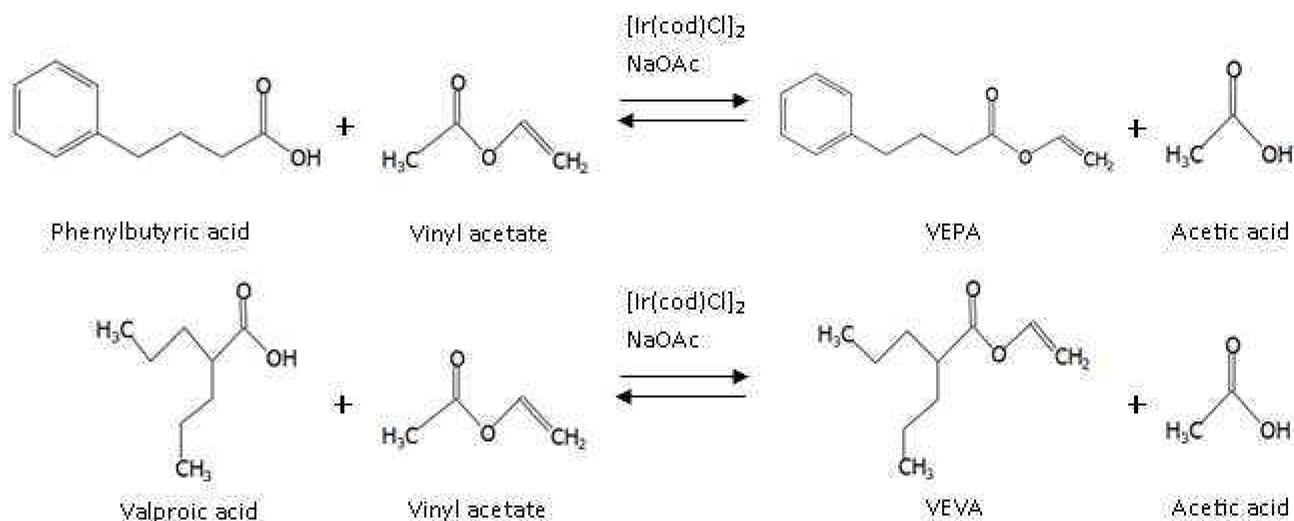



Figure 4: Catalysts bis(1,5-cyclooctadiene)diiridium(I) dichloride ([Ir(cod)Cl]2) and sodium acetate (NaOAc).

iridium complex bis(1,5-cyclooctadiene)diiridium(I) dichloride ($[Ir(cod)Cl]_2$) in the reaction of vinyl acetate with several carboxylic acids (17). In a typical reaction, carboxylic acid was reacted with 10 eq vinyl acetate in the presence of 1 mol% $[Ir(cod)Cl]_2$ and 3 mol% sodium acetate (NaOAc) (Figure 4) in toluene at 100 °C for 15 hr under argon atmosphere. Running the reaction without NaOAc lowered the % conversion. Reacting cinnamic acid with vinyl acetate resulted in 80% conversion. An excess of vinyl acetate is used in the reaction in order to push the equilibrium toward the product side.

Figure 5 describes the scheme for the reaction of phenylbutyric acid and valproic acid

Figure 5: Reaction scheme for the synthesis of VEPA and VEVA from their carboxylic acid precursors.



with vinyl acetate to form corresponding vinyl esters. Table 1 lists the conditions of each reaction performed and their associated % yield. Phenylbutyric acid or valproic acid was reacted with 10 eq (later 20 eq) of vinyl acetate in the presence of 1 mol% $[Ir(cod)Cl]_2$ and 3 mol% NaOAc refluxed at 100 °C in toluene for 15 hr under nitrogen. Consumption of the carboxylic acid at the end of 15 hr was verified by thin layer chromatography (TLC). TLC was run with 4:1 (VEPA $R_f$ = 0.78) or 10:1 (VEPA $R_f$ = 0.49) hexanes:ethyl acetate and visualized with either Seebach's stain (VEPA) or iodine vapor (VEVA). Product was concentrated on rotary evaporator and vacuum filtered with 5 µm filter paper. Purification was performed by column

**Table 1: Reaction conditions for the synthesis of VEPA and VEVA.**

| Carboxylic acid | Vinyl acetate | Solvent | Reaction time | % yield |
|---|---|---|---|---|
| 1 mmol PBA | 10 eq | 3 mL toluene | 15 hr | 47 |
| 10 mmol PBA | 20 eq | 30 mL toluene | 15 hr | 86 |
| 7.4 mmol PBA | 20 eq | 22 mL toluene | 20 hr | 88 |
| 15 mmol PBA | 20 eq | 45 mL toluene | 16 hr | 97 |
| 15 mmol PBA | 20 eq | 45 mL toluene | 16 hr | 93 |
| 10 mmol VPA | 20 eq | 30 mL toluene | 15 hr | 20 |
| 10 mmol VPA | 20 eq | 30 mL toluene | 15 hr | 71 |
| 19.7 mmol VPA | 20 eq | 59 mL toluene | 15 hr | 73 |
| 19.7 mmol VPA | 20 eq | 59 mL toluene | 15 hr | 69 |

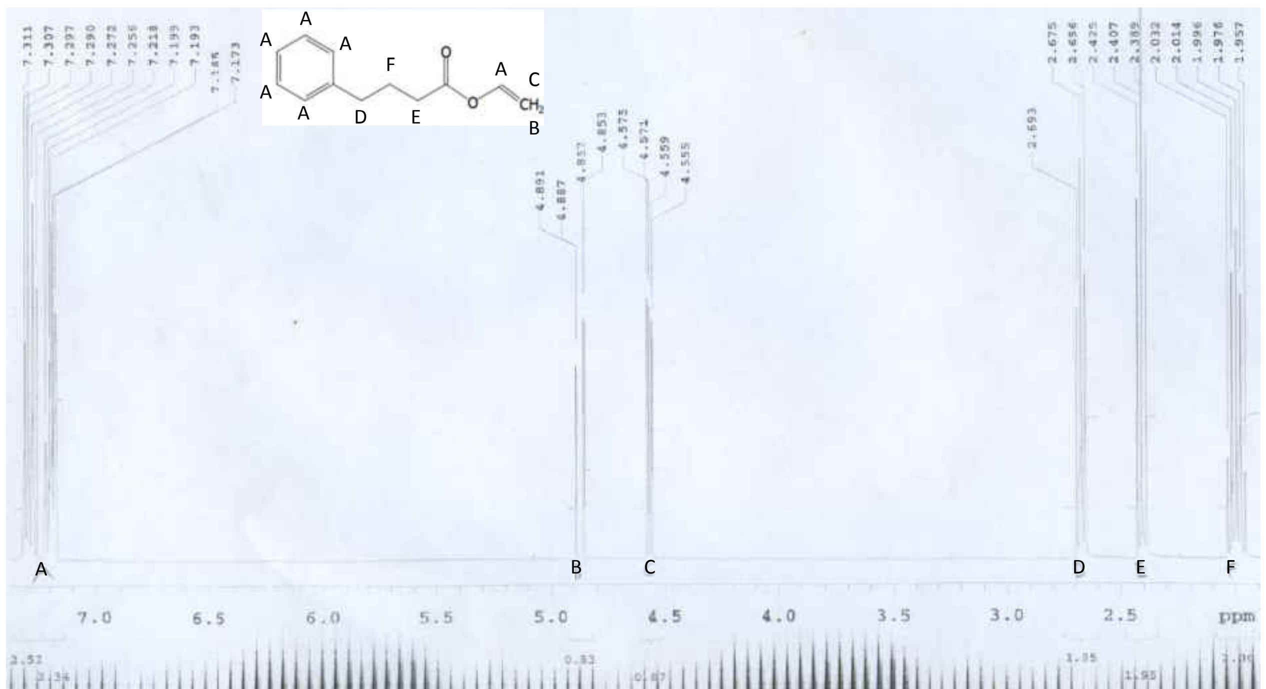



Figure 6: Proton NMR spectra of VEPA in chloroform.

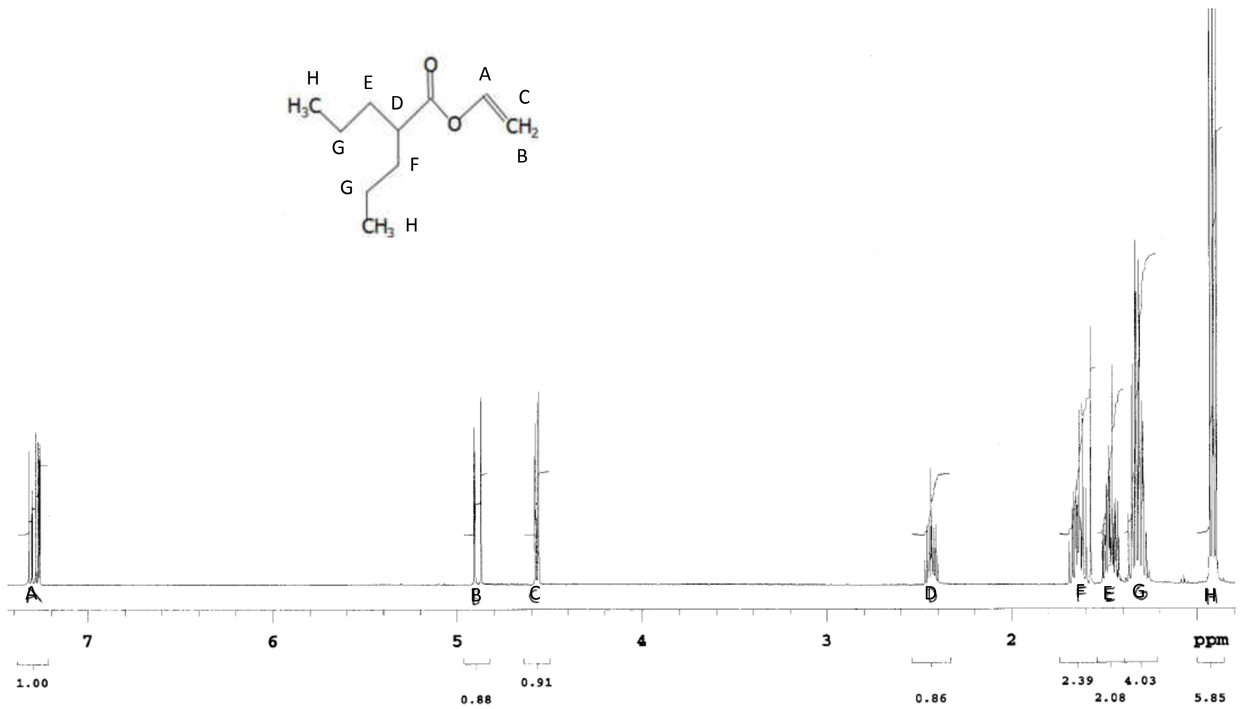



Figure 7: Proton NMR spectra of VEVA in chloroform.

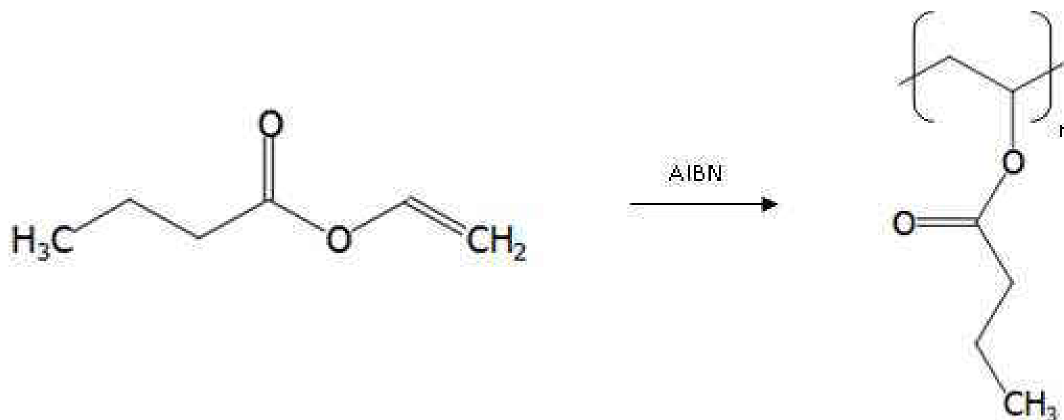



Figure 8: Free radical polymerization of vinyl butyrate.

chromatography using 20:1 hexanes:ethyl acetate. VEVA was markedly more volatile than VEPA; initial concentration of VEVA on rotary evaporator was thus performed at lower bath temperature and run twice in order to minimize evaporation of the monomer. Unfamiliarity with the volatility of VEVA led to low % yield initially. Adding additional excess of vinyl acetate to the reactants improved the % yield. VEPA and VEVA appear as faintly yellowish transparent oils and are soluble in methanol, acetone, hexanes and ethyl acetate but insoluble in water.

Identity and purity of VEPA and VEVA monomers were verified by proton NMR in chloroform (Figure 6, Figure 7). The only example of VEPA synthesis found in the literature (18) includes NMR data that were successfully replicated here. Identification of VEVA by NMR was facilitated with published NMR spectra of VPA. NMR peaks of both monomers were clean and distinct indicating a successful purification and suitability for use in polymerization studies.

*Poly(vinyl butyrate) as test case*

In order to optimize and practice polymerization methods prior to polymerizing VEPA and VEVA monomers, a commercially available vinyl ester of similar structure was examined. Vinyl butyrate was polymerized by AIBN to form poly(vinyl butyrate) (Figure 8). There are a few common polymer manufacturing techniques to choose from:

- Bulk polymerization, a reaction of monomer and initiator only
- Solution polymerization, a reaction of monomer and initiator dissolved in a solvent
- Suspension polymerization, a reaction consisting of a liquid phase and a mechanically agitated oil phase of suspended monomer droplets

Solution polymerization was chosen as the synthetic method here because the reaction mixture has a lower viscosity than that of bulk polymerization. Lower viscosity facilitates a more uniform heat distribution throughout the polymerization mixture and reduces the molecular weight polydispersity of the obtained polymers (19). Suspension polymerization was decided against due to the added complexity introduced by its high dependence on stirring rate.

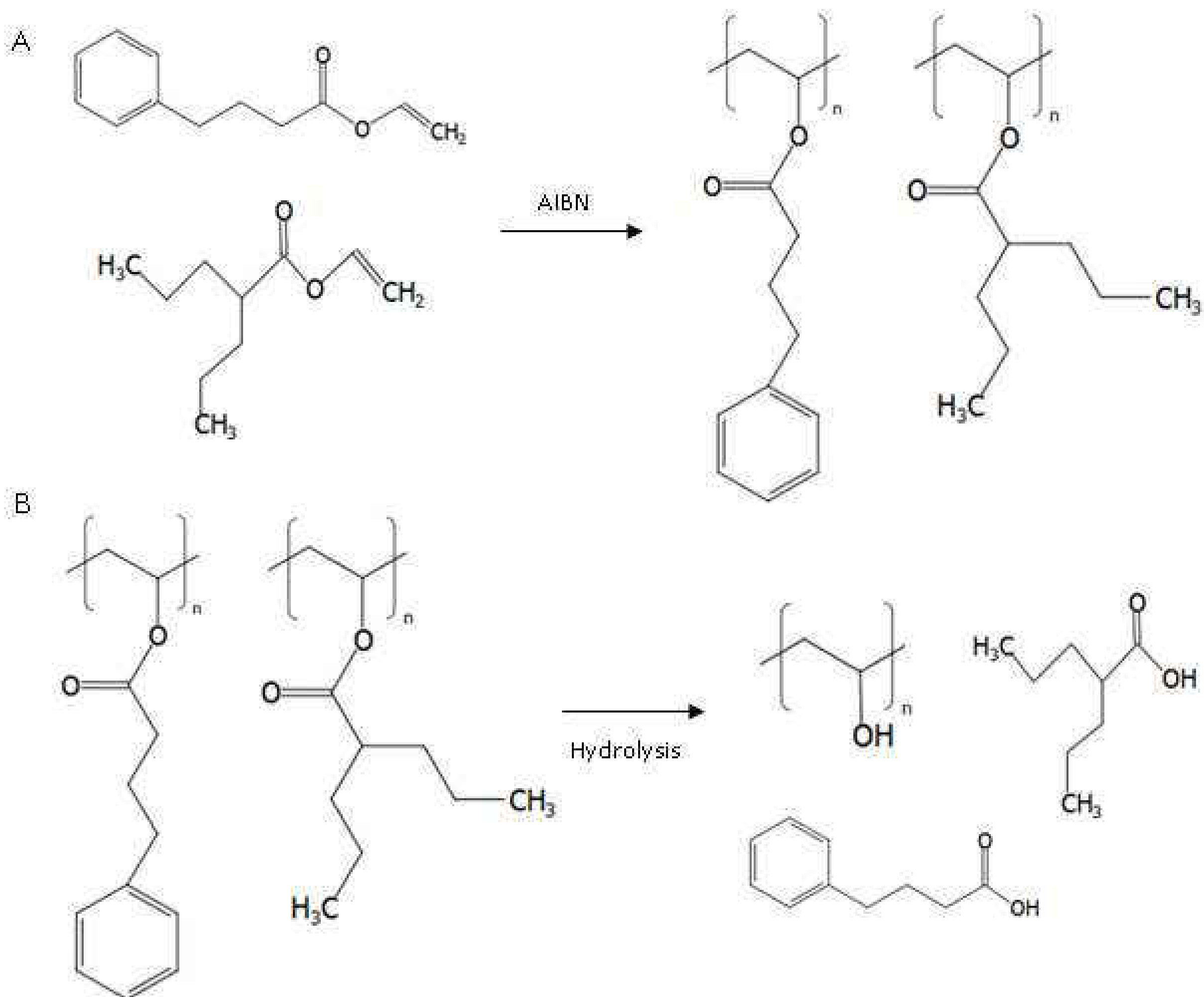
After being vacuum distilled to remove stabilizer, vinyl butyrate was polymerized in stirred DMF at 60°C in a water bath for 24 hr under nitrogen. Precipitation of the polymer was initially attempted in chilled ether but was unsuccessful. Instead, the reaction mixture was poured in chilled distilled water, resulting in a polymer precipitate, which was subsequently

**Table 2: Reaction conditions for the polymerization of vinyl butyrate.**

| Monomer | Initiator | Solvent | Temperature | Reaction time | Molecular weight |
|---|---|---|---|---|---|
| 1 g vinyl butyrate | 14 mg AIBN | 4 g DMF | 60°C | 24 hr | 7073 |
| 1 g vinyl butyrate | 14 mg AIBN | 4 g DMF | 60°C | 48 hr | 4800 |
| 1 g vinyl butyrate | 7 mg AIBN | 4 g DMF | 60°C | 48 hr | 4350 |
| 1 g vinyl butyrate | 2.5 mg AIBN | 4 g DMF | 60°C | 48 hr | 4890 |
| 1 g vinyl butyrate | 14 mg AIBN | 4 g acetone | 60°C | 48 hr | 11157 |

dried under vacuum at room temperature for 24 hr. The initial poly(vinyl butyrate) sample was found to be soluble in DMF, methanol, acetone, toluene, dichloromethane and THF, but insoluble in water. A molecular weight of 7073 as measured by gel permeation chromatography (GPC) was considered low, so the concentration of initiator was reduced in the following trials in hopes of raising the molecular weight, but no trend was noticeable within the used initiator concentration range (Table 2). Differential scanning calorimetry (DSC) performed on several poly(vinyl butyrate) samples identified them as amorphous without a crystallization temperature peak. Change in solvent to acetone from DMF produced a markedly higher

**Figure 9: Synthesis and degradation of poly(VEPA) and poly(VEVA). A: Polymerization of VEPA and VEVA monomer. B: Degradation of poly(VEPA) and poly(VEVA) into poly(vinyl alcohol) and their respective carboxylic acids.**



molecular weight, so polymerization in acetone under conditions stated in Table 2 was chosen to be the starting point for VEPA and VEVA polymerization.

*Polymerization of VEPA and VEVA*

Figure 9 describes the polymerization of VEPA and VEVA by the addition of the free radical initiator AIBN. Poly(VEPA) and poly(VEVA) take the form of polyvinyl alcohol chains with HDAC inhibitors attached to the chains as pendant groups by their esters. Ideally, chemical or enzymatic hydrolysis will attack these esters and result in polyvinyl alcohol (which is nontoxic and generally recognized as safe (GRAS) by the FDA) and HDAC inhibitor carboxylic acids. Table 3 lists the conditions of poly(VEPA) and poly(VEVA) polymerizations conducted. Conditions

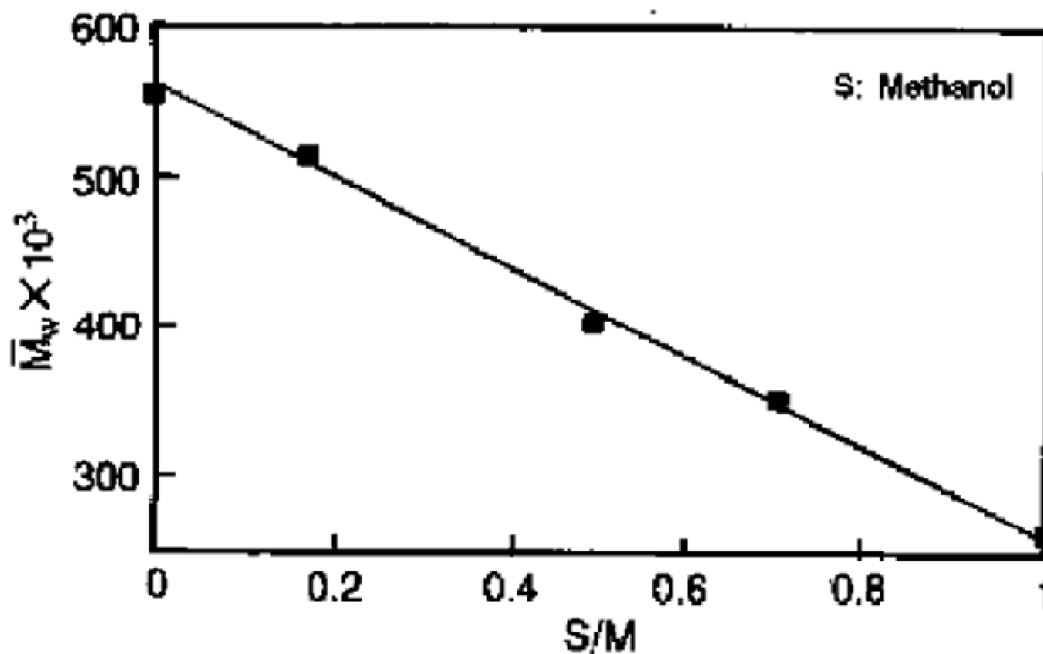
Table 3: Poly(VEPA) and poly(VEVA) polymerization and precipitation conditions.

| Monomer | Initiator (% weight) | Solvent | Heat | Reaction time | Dropped in | Precipitated? (Y/N) |
|---|---|---|---|---|---|---|
| 125mg VEPA | 0.4mg AIBN (0.3%) | 0.5g acetone | 60° C | 48 hr | Ether, water | N |
| 25mg VEPA | 1mg AIBN (4%) | 3mL benzene | 60° C | 48 hr | Ether | N |
| 25mg VEPA | 10mg AIBN (40%) | 3mL benzene | 60° C | 48 hr | Ether | N |
| 25mg VEPA | 25mg AIBN (100%) | 3mL benzene | 60° C | 48 hr | Ether | N |
| 25mg VEPA | 50mg AIBN (200%) | 3mL benzene | 60° C | 48 hr | Ether | N |
| 25mg VEPA | 2.5mg AIBN (10%) | 2mL methanol | 60° C | 48 hr | Acetone, ether, water | N |
| 25mg vinyl acetate | 2.5mg AIBN (10%) | 2mL benzene | 60° C | 48 hr | Hexanes, ether, water | N |
| 100mg VEPA | 2mg AIBN (2%) | 100mg methanol | 60° C | 48 hr | Methanol, hexanes | Y |
| 2.5g VEPA | 50mg AIBN (2%) | 2.5g methanol | 60° C | 48 hr | Methanol, hexanes | Y |
| 2.5g VEVA | 50mg AIBN (2%) | 2.5g methanol | 60° C | 48 hr | Methanol, hexanes | Y |

matching that of the final poly(vinyl butyrate) polymerization were first attempted. When that proved unsuccessful, the amount of initiator was varied in order to rule out that insufficient amount of initiator limited the polymerization. Benzene and methanol were substituted for acetone in an attempt to reduce chain transfer to solvent. Finally, vinyl acetate was substituted for VEPA monomer. Vinyl acetate is commonly produced by free radical solution polymerization, so its failure to polymerize under these conditions denoted a severe flaw in the procedure.

A literature search uncovered a study (20) that examined the molecular weight of poly(vinyl acetate) when polymerized in methanol solution with varying solvent/monomer weight ratios (Figure 10). An inverse relationship between solvent/monomer ratio and molecular weight was found. Given that the poly(VEPA) solvent/monomer ratios attempted far exceeded that of (20), further polymerizations were instead conducted at a solvent/monomer

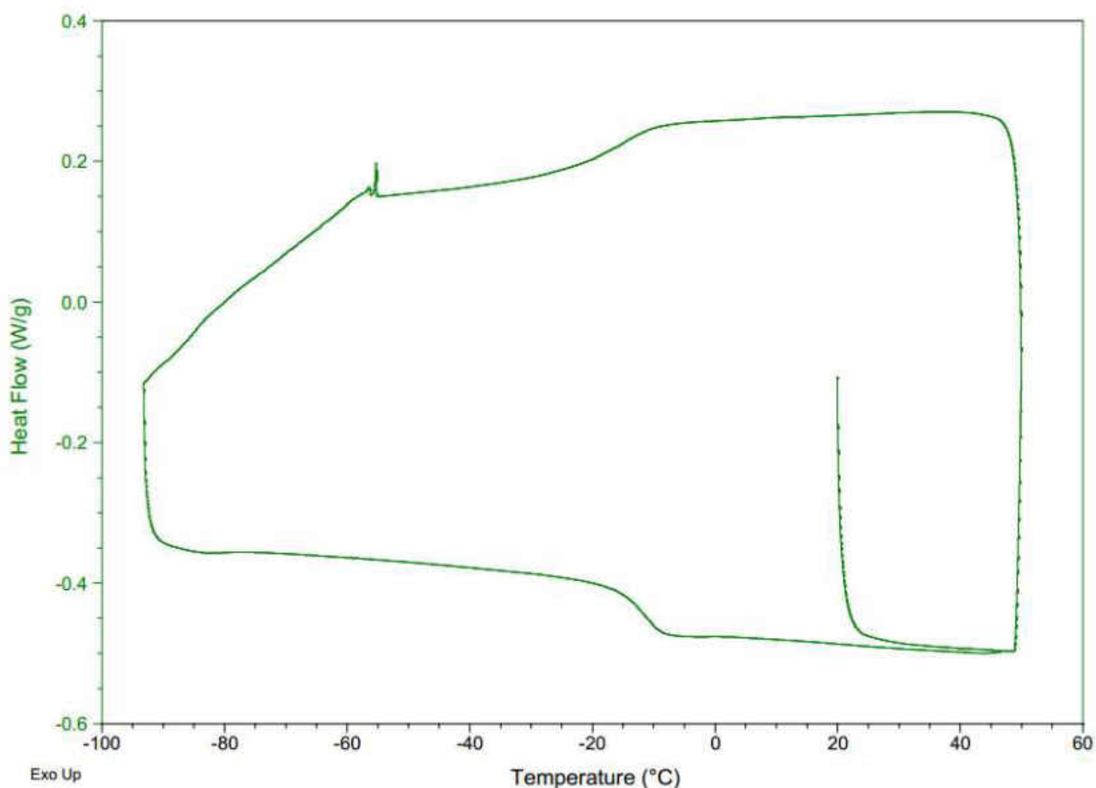
**Figure 10: Effect of varied solvent/monomer weight ratio on molecular weight in the solution polymerization of poly(vinyl acetate). Figure attributed to (20).**



ratio of 1. A representative small-scale polymerization procedure involved 100 mg VEPA, 100 mg methanol and 2 mg AIBN (2% wt) heated under nitrogen in an oil bath at 60°C for 48 hr. As polymerization progressed the homogenous reaction mixture formed two distinct layers – a layer of viscous white polymer and a layer of solvent above – making it more accurately termed a precipitation polymerization. The excess solvent could be pipetted away, and the impure polymer was found to be insoluble in methanol, hexanes and water.

Precipitation of gram-scale polymers was carried out by dissolving the impure polymer in 2x volume dichloromethane (DCM) and adding dropwise into 200 mL stirred methanol. Polymer flakes formed but the majority of precipitated polymer accumulated on magnetic stirrer bar and beaker walls as a sticky white solid. The milky white precipitation solution was decanted into 50 mL Falcon tubes and centrifuged down at 4000 rpm for 10 min, then decanted

**Figure 11: Differential Scanning Calorimetry (DSC) data taken from a sample of poly(VEPA).**



again. The polymer remaining in the centrifuge tube as well as the polymer accumulated in the precipitation flask was redissolved in DCM. Polymer was purified by repeating this precipitation procedure once more. Polymer solution was concentrated by rotary evaporator and dried under vacuum at room temperature for 24 hr.

Poly(VEPA) and poly(VEVA) polymers were clear, soft, stringy and gel-like at room temperature. Thus, it was expected that these polymers were amorphous. DSC data taken from a poly(VEPA) sample demonstrated a glass transition temperature (Tg) of -12.5°C and no detectable crystallization peak (Figure 11). GPC conducted on a poly(VEPA) sample gave a weight-average molecular weight of 25,800 and PDI of 2.43. Also investigated was the possibility of synthesizing electrospun films from these polymers. At the experimental scales shown in Table 3, a poly(VEPA) or poly(VEVA) solution dried to sufficient viscosity for electrospinning was beneath the minimum required volume for a film to be produced. The amorphous and sticky nature of the polymer in bulk form made it difficult to work with and incurred material losses when transferring it between vessels. Though unoptimized for handling characteristics and/or a higher glass transition temperature, the successful polymerization of VEPA and VEVA fulfilled the second specific aim stated in Chapter 1.

*Direct esterification of poly(vinyl alcohol) with PBA*

The reaction of acid chlorides with polyvinyl alcohol (PVA) leads to poly(vinyl esters) (21). Hence, instead of polymerizing VEPA and VEVA we have utilized preformed PVA and reacted the available hydroxyl groups with corresponding acid chloride. The reaction scheme for this approach is given in Figure 12. PBA was reacted with 1.1 eq thionyl chloride and a catalytic amount of DMF in THF or chloroform under nitrogen atmosphere. The reactants were

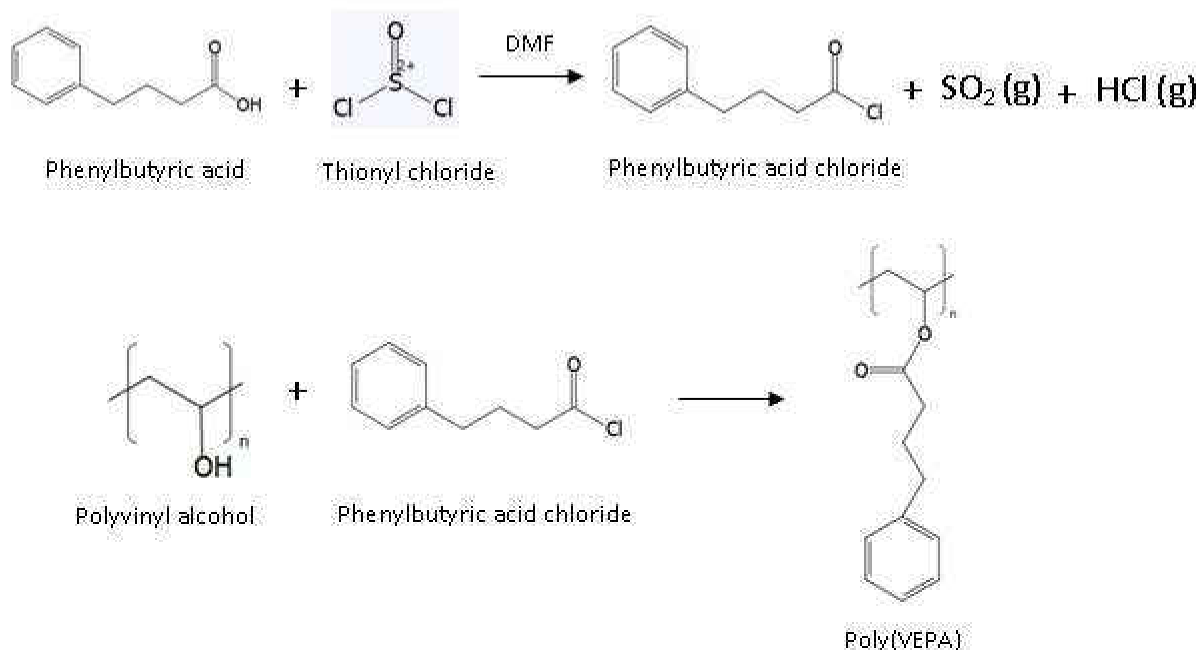



**Figure 12: Reaction scheme for the direct esterification of poly(vinyl alcohol) with PBA.**

brought to reflux and reacted for 3 hr to form phenylbutyric acid chloride (PBACl). PVA was reacted with 2.5 eq of PBACl under nitrogen atmosphere at reflux. PVA was of either 1.5% acetate or 10% acetate. Reaction times of 2 hr and overnight were attempted. When left overnight the PBACl solution turned from yellow to a dark brownish-black. The majority of PVA remained un-dissolved in PBACl solution including the more reactive 10% acetate variety of PVA. The reaction mixtures were passed through a PTFE disc filter and added dropwise in stirred hexanes but no precipitation occurred. Esterification of poly(vinyl alcohol) with PBA was not found to be a practical means of preparing poly(VEPA) under the conditions examined here. It is possible future studies may have more success in utilizing PVA of different purity or a different reaction mixture more capable of solubilizing PVA.

# CHAPTER 3: MICROPARTICLE PREPARATION AND CHARACTERIZATION

*Microparticle preparation*

Microparticles and nanoparticles prepared from bioactive polymers are an established means of drug delivery (22). Microparticles can facilitate cell-induced aggregation and may be sintered together to form porous scaffolds for use in tissue engineering and regenerative medicine applications (23). Poly(VEPA) and poly(VEVA) microparticles were investigated for their ease of use as a tool in the evaluation of polymer degradation and for their potential as a scaffold material in future studies.

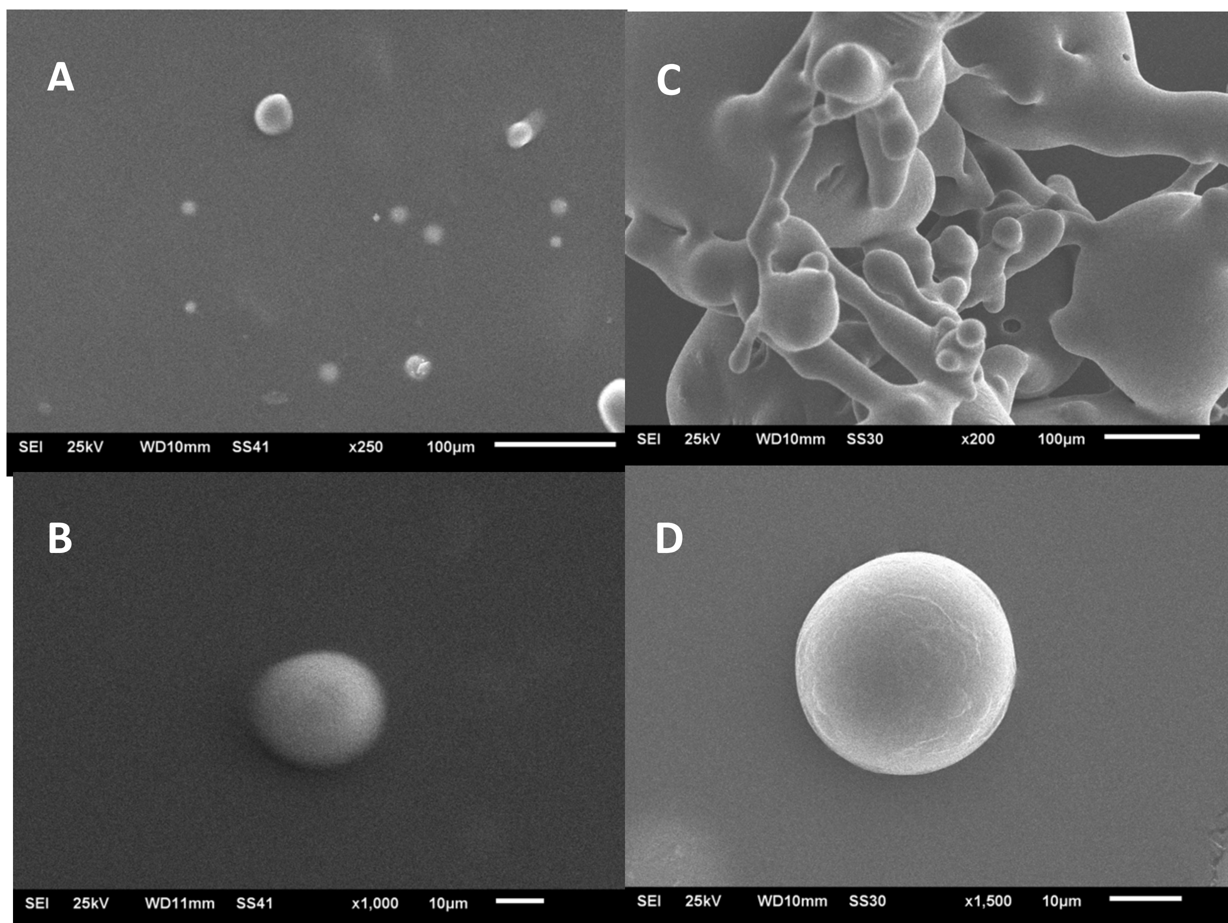
Microparticles were prepared by an emulsion-solvent evaporation method with a typical procedure as follows: 50-100 mg of polymer was dissolved in 2 ml dichloromethane and dropped in 6-8 ml of 1% PVA, then emulsified with a homogenizer at 30,000 rpm for 5 minutes while chilled in an ice bath. Emulsified solution was poured into 200 ml 0.1% PVA and slowly stirred for 1 hour (poly(VEVA)) or 3 hours (poly(VEPA)). Microparticles were isolated by centrifugation at 4000 rpm for 10 minutes (poly(VEPA)) or 20,000 rpm for 40 minutes (poly(VEVA)), redispersed by sonication and vortexing and washed once with DI water (poly(VEPA) only). It was found that when poly(VEVA) particles were prepared under the same conditions as poly(VEPA), the poly(VEVA) particles could not be pelleted during centrifugation. Particles were then suspended in 10 ml DI water, frozen in a -80°C freezer for 1 hr and lyophilized for 48 hr. These lyophilized microparticles took the form of a flat, sticky paste that could not be resuspended in solution. When 5% sucrose, a common cryoprotectant (24), was added to the pre-freeze suspension, the final lyophilized product took the form of a dry white

fluffy powder. The powder form was a desirable product because it facilitated easy measurement of microparticles and a stable shelf life when frozen.

*Microparticle characterization*

Mixed results were obtained when attempting to resuspend sucrose-protected lyophilized microparticles. When thoroughly sonicated and vortexed after centrifugation, poly(VEPA) particles could be resuspended 4-5 times in water before the pellet seized up. Poly(VEVA) particles could be resuspended once in water but proved resistant to further centrifugation, even at upwards of 40,000 rpm for 1 hr. Scanning electron microscopy (SEM) images (Figure 13) taken of poly(VEPA) and poly(VEVA) microparticles show sections of discrete

**Figure 13: Scanning electron micrograph of poly(VEPA) (A,B) and poly(VEVA) (C,D) microparticle samples.**



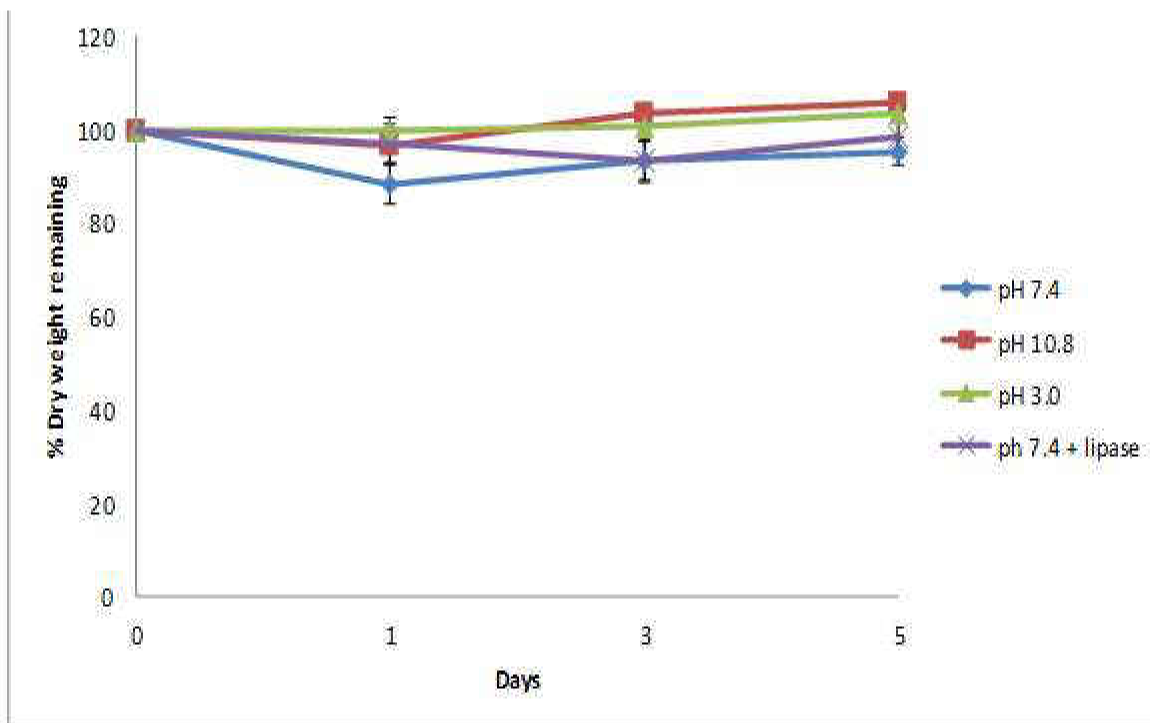
spheres in the 20-40 µm diameter range. Larger masses of agglomerated polymer with sphere-like projections were also present in samples examined. Poly(VEPA) and poly(VEVA) microparticles prepared did fulfill the third specific aim listed in Chapter 1, but their size was not very uniform and significant agglomeration occurred. Greater consistency in microparticle morphology would make them a more attractive scaffold material.

# CHAPTER 4: EVALUATION OF POLYMER DEGRADATION

*Degradation mechanisms*

Poly(VEPA) and poly(VEVA) were expected to degrade in two ways. In chemical hydrolysis, hydrolytic scission of the ester groups separates the pendant carboxylic acids VEPA and VEVA from their polyvinyl alcohol chain with the addition of water. In enzymatic hydrolysis, the scission of ester groups is catalyzed by the addition of an enzyme such as lipase (25). Polyvinyl alcohol has low cytotoxicity and carries generally recognized as safe (GRAS) status making it an attractive degradation product. As these polymers were not tuned to specifically undergo surface erosion, an assumption was made that they would undergo the more commonly observed bulk erosion (26). Because bulk erosion is not dependent on surface area of a sample, results of hydrolysis studies conducted on polymer particles were representative of the polymer in bulk.

Figure 14: Hydrolytic degradation of poly(VEPA).



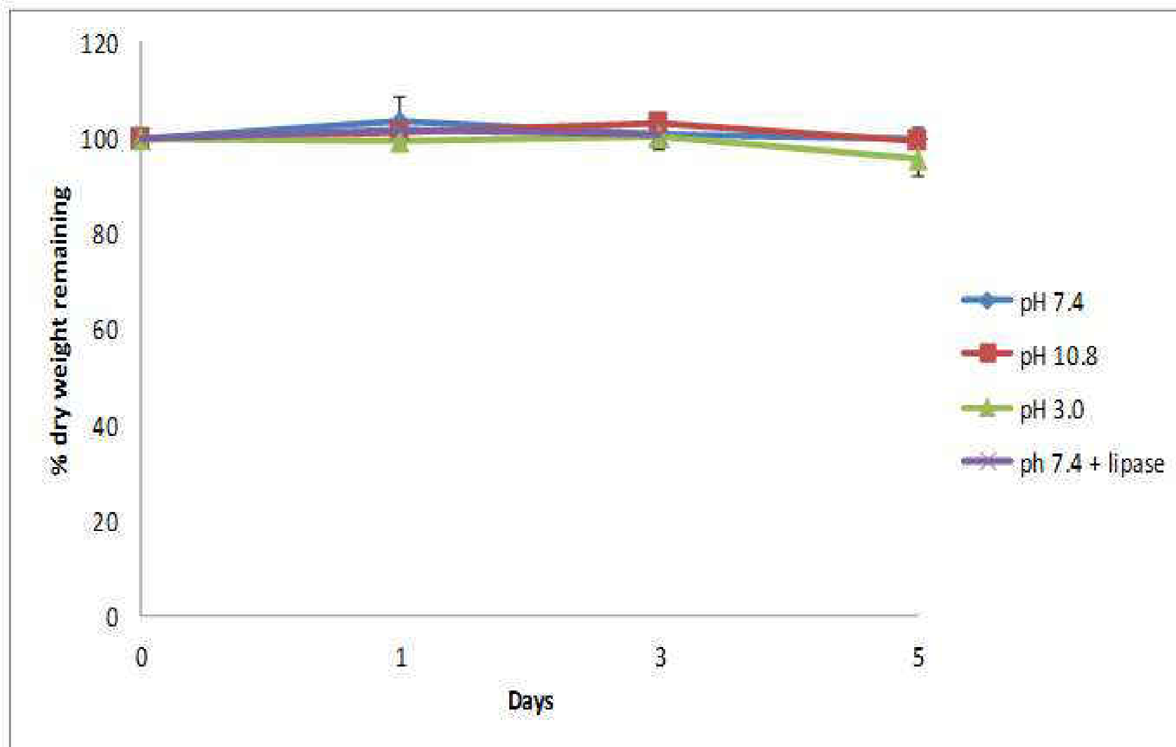



**Figure 15: Hydrolytic degradation of poly(VEVA).**

*Evaluation of chemical and enzymatic hydrolysis*

Hydrolytic degradation was evaluated by examination of dry weight loss and isolation of degradation products. In the dry weight loss experiment, samples of 3-5mg poly(VEPA) and poly(VEVA) particles were measured into small centrifuge tubes and dispersed in 1 ml of each of four freshly prepared solutions: phosphate buffered saline (PBS) at pH 7.4, citric acid/trisodium citrate buffer at pH 3.0, sodium carbonate/sodium bicarbonate buffer at pH 10.8, and lipase from *Candida antarctica* (1.16 U/mg) at 0.1 mg/ml in PBS at pH 7.4. The amount of lipase applied falls within the reference range of concentration in human serum (25). Samples were prepared in triplicate. Samples were placed in a 37°C shaker and taken out at time points of 1, 3 and 5 days. A set of samples at each time point were washed with DI water and dried under vacuum at room temperature for 24 hr, while remaining samples were

centrifuged, decanted and filled with fresh buffer before being returned to incubation. Samples were weighed before incubation and after drying to calculate dry % weight loss. Results are displayed in Figures 14 and 15. No significant degradation of polymer was detected by measure of dry weight loss.

Isolation of degradation products was investigated by the use of high-performance liquid chromatography (HPLC). Because valproic acid could not be measured by the UV detector present within HPLC equipment, only hydrolysis of poly(VEPA) was examined. Samples (in triplicate) of 10-15mg of poly(VEPA) particles were dispersed in PBS at pH 7.4, citric acid/sodium tricitrate buffer at pH 3.0 and sodium carbonate/sodium bicarbonate buffer at pH 10.8. Samples were incubated in a 37°C shaker for 9 days. At day 1 and every 2 days following samples were removed from the shaker, centrifuged, decanted into vials for analysis and replaced with fresh buffer. An HPLC concentration curve for PBA was prepared by analyzing solutions of PBA in HPLC water. Sample solutions of acidic or basic pH were neutralized for compatibility with the HPLC column by dropwise addition of HCl or $Na_2CO_3$, respectively. HPLC analysis conducted on these samples demonstrated a nearly undetectable quantity of PBA present (data not shown). In an attempt to extract more degradation products, a separate sample of poly(VEPA) particles dispersed in PBS at pH 7.4 was kept in a block incubator for 15 hr at 65°C. Given the experimental value obtained for molecular weight of poly(VEPA), ideal release of PBA could be calculated. When analyzed for PBA content (Figure 16), the sample contained only 3.7% of the expected quantity for 100% hydrolysis. HPLC and dry weight loss results both provide evidence for the claim that no substantial degradation of polymer by hydrolysis took place *in vitro*.

*MTT assay*

An MTT assay was used to evaluate the cytotoxicity of poly(VEPA) and poly(VEVA) microparticles. HeLa cells were seeded in 96 well culture plates and cultured to confluency in Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Five sample groups in triplicate were added to the cell culture plate in doses of 10, 50, 100, 200, 400, and 800 µg/ml. Sample groups were poly(VEPA) microparticles, poly(VEVA) microparticles, polyvinyl alcohol (10% acetate), PBA and VPA. The dose range examined was consistent with an MTT assay of VPA (27) and applications of VPA (11) and PBA (5) reported in the literature. After 24 hours of incubation at 37°C the cells were washed with PBS and MTT assay was conducted (Figure 17).

The results of the MTT assay were inconsistent with expectations. Poly(VEPA) and poly(VEVA) microparticles were found to promote cell growth and not inhibit it. The cause was found to be residual cryoprotective sucrose from the lyophilization process. A new MTT assay for microparticle samples was conducted under the same conditions, but instead of direct dispersion into culture medium the microparticles were washed once with water and once with ethanol before dispersion. However, as previously mentioned poly(VEVA) particles could not be centrifuged successfully in water under any centrifugation conditions attempted (upwards of 40,000 rpm for 1 hour). Instead, an MTT assay was run solely on washed poly(VEPA) particles (Figure 18). Poly(VEPA) particles without sucrose were found to cause no significant cytoxicity to HeLa cells. To cause no significant cell death among HeLa cells *in vitro* is a further demonstration that poly(VEPA) is not vulnerable to hydrolysis under physiological conditions and is thus ineffective as a drug delivery scaffold material.

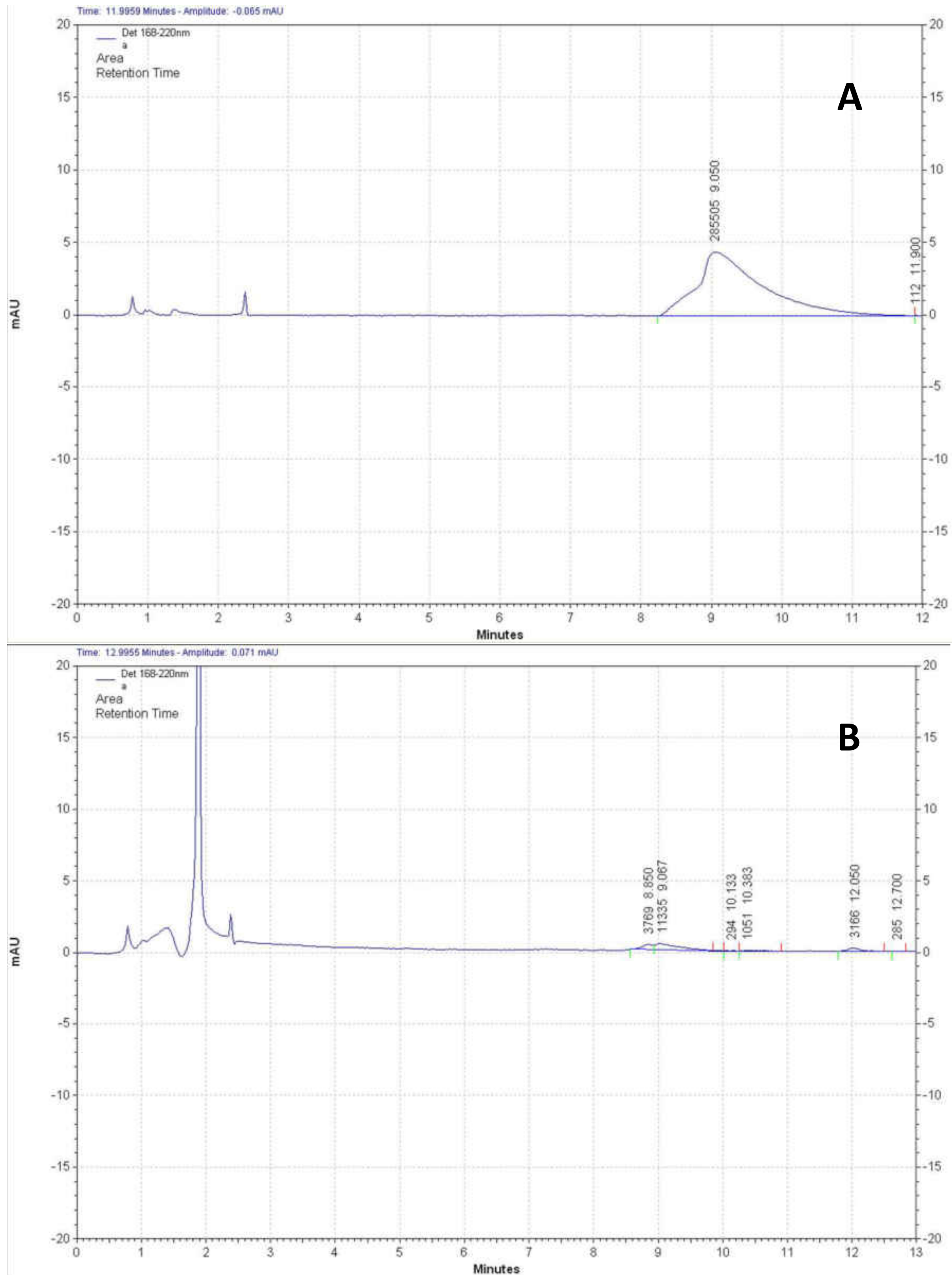



**Figure 16: HPLC analysis of hydrolyzed poly(VEPA) microparticles. (A) Calibration solution of 25 µg PBA in HPLC water. (B) 8.9 mg Poly(VEPA) microparticles incubated for 15 hr at 65°C in PBS.**

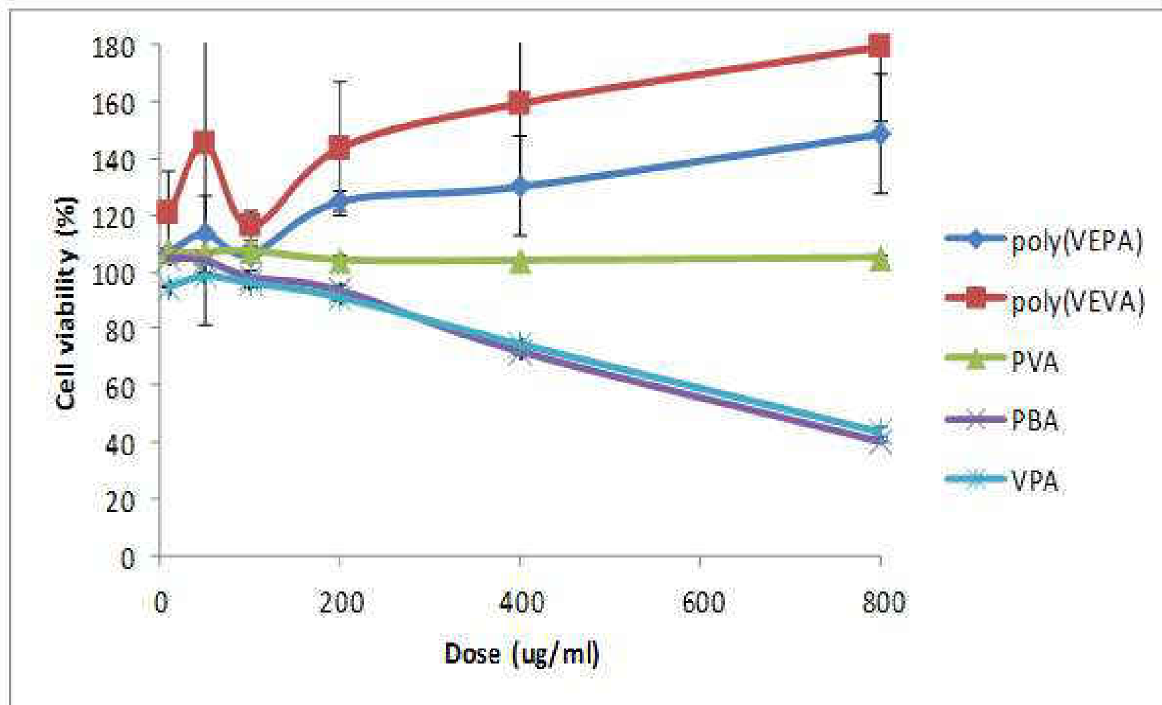



Figure 17: Evaluation of cytotoxicity of sucrose-adulterated poly(VEPA) and poly(VEVA) microparticles on HeLa cells by MTT assay.

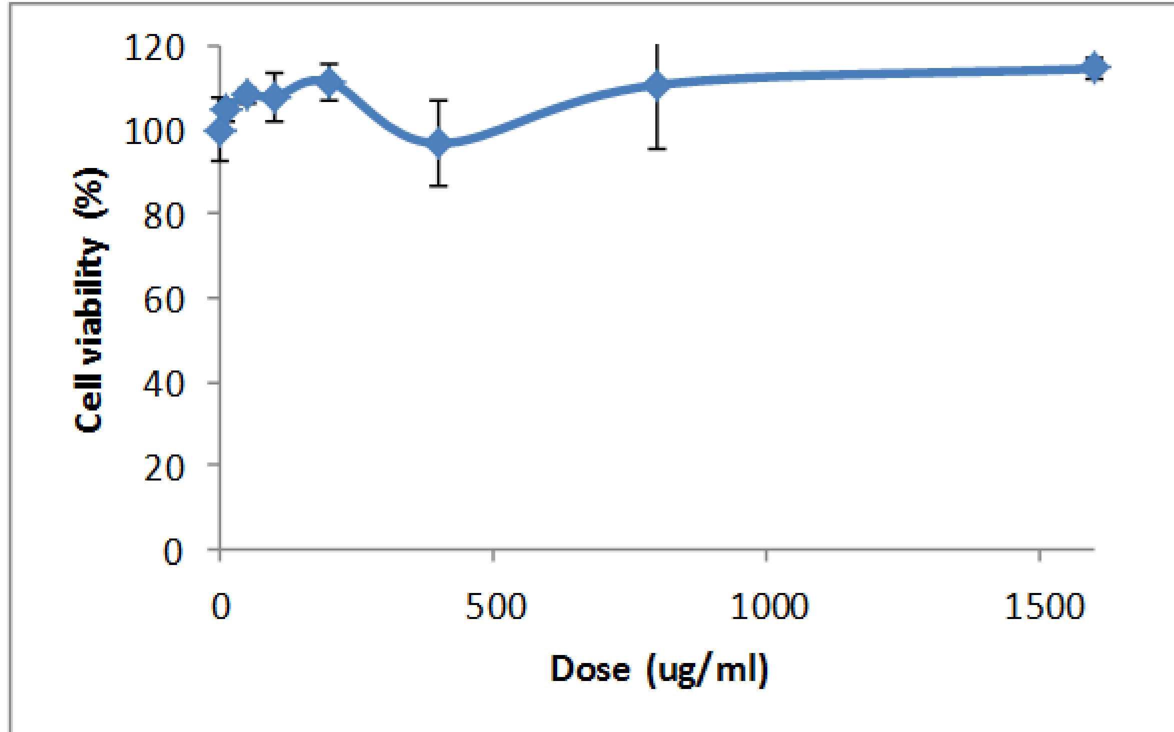



Figure 18: Evaluation of cytotoxicity of sucrose-free poly(VEPA) microparticles on HeLa cells by MTT assay.

# CHAPTER 5: CONCLUSION

In this study, valproic acid and phenylbutyric acid-based vinyl polymers were successfully prepared from iridium complex-catalyzed vinyl ester monomers by free radical polymerization. Polymerization by means of reaction between phenylbutyric acid chloride or valproic acid chloride and polyvinyl alcohol was unsuccessful due to insufficient solubility of polyvinyl alcohol in reaction medium. Poly(VEPA) and poly(VEVA) microparticles on the 20-40 µm diameter scale were successfully prepared from bulk polymer and verified by SEM, but poly(VEVA) microparticles proved incapable of withstanding any wash cycles. Ultimately these polymers failed to exhibit the necessary properties for their use as a biodegradable scaffold material in the generation of iPSCs or as an anti-inflammatory agent. Examination of hydrolysis at varied pH and with the inclusion of lipase by dry weight loss and HPLC failed to produce evidence of any significant degradation of polymer under a timeframe suitable for iPSC generation. An assay that would examine the HDAC inhibition of HeLa cells in the presence of poly(VEPA) and poly(VEVA) microparticles was planned for but determined to be unnecessary in light of the conclusiveness of other findings. It is proposed that the addition of bulky and/or phenyl-containing pendant groups may have contributed to an overly hydrophobic nature of polymer and protected the esters from hydrolytic attack. If future studies examine polymers derived from these HDAC inhibitors it is advisable to link them to a polymer backbone with a bond more susceptible to short-term degradation.

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## ABSTRACT

**SYNTHESIS AND CHARACTERIZATION OF BIODEGRADABLE POLY(VINYL ESTERS) WITH HDAC INHIBITORY ACTIVITY**

by

**KYLE L. HORTON**

**May 2013**

**Advisor:** Dr. David Oupicky

**Major:** Biomedical Engineering

**Degree:** Master of Science

HDAC inhibitors are known to have anti-inflammatory properties. HDAC inhibitors are used in combination with Oct4 to generate induced pluripotent stem cells. I hypothesized that polyesters based on simple aliphatic HDAC inhibitors like valproic acid (VPA) and phenylbutyric acid (PBA) can serve as alternatives to existing polyester biomaterials with improved anti-inflammatory properties and as scaffolds for generation of iPSCs when used in combination with layer-by-layer thin films delivering reprogramming transcription factors. Vinyl ester of phenylbutyric acid (VEPA) and vinyl ester of valproic acid (VEVA) were synthesized from their carboxylic acid precursors using an iridium complex catalyst at yields as high as 97% and 73%, respectively. Amorphous poly(VEPA) and poly(VEVA) polymers were prepared by free radical solution polymerization and characterized for molecular weight and glass transition temperature. Poly(VEPA) and poly(VEVA) microparticles of 20-40 µm diameter were prepared by an emulsion-solvent evaporation method and examined under scanning electron microscopy (SEM). Their hydrolytic degradation was studied by dry weight loss and HDAC inhibitor release *via* high performance liquid chromatography (HPLC) in the presence of varied pH and lipase-

containing buffers. No significant degradation occurred within 5 days, and an MTT assay conducted on HeLa cells in the presence of these microparticles confirmed an absence of cytotoxicity. Poly(VEPA) and poly(VEVA) microparticles were not found to be a suitable biomaterial for hypothesized applications in light of their poor degradation characteristics *in vitro*.

## AUTOBIOGRAPHICAL STATEMENT

Kyle L. Horton, B.S., EIT received his bachelor's degree in biomedical engineering in 2009 from Rose-Hulman Institute of Technology in Terre Haute, IN. While a student there, he passed the Fundamentals of Engineering (FE) exam and he and his capstone project team received the Best Undergraduate Student Poster Award at the 2009 American Society for Engineering Education (ASEE) IL/IN section conference for their development of a concussion sensor prototype in football helmets.

After obtaining his bachelor's degree, in 2010 he moved to Detroit, MI to attend Wayne State University as a master's student in biomedical engineering. He joined the lab of Dr. David Oupicky in 2011. His research interests lie in the development of induced pluripotent stem cells (iPSCs) and the study of endothelial progenitor cells (EPCs). He plans to enter the medical device, biologics or pharmaceuticals industry upon graduation.