CALIFORNIA POLYTECHNIC STATE UNIVERSITY, SAN LUIS OBISPO

SENIOR PROJECT

Preparation and Characterization of EVA/CORT Microparticles for Rattlesnake Endocrinology Studies

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"Once you can accept the universe as matter expanding into nothing that is something, wearing stripes with plaid comes easy."

Albert Einstein

CALIFORNIA POLYTECHNIC STATE UNIVERSITY, SAN LUIS OBISPO

Abstract

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Bachelor of Science

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by Alexander PAUER

In conservation physiology, a common difficulty in assessing the effects of the stress response in animals is maintaining consistent elevation of stress hormones. It has become a goal of researchers to experimentally elevate the stress hormone corticosterone (CORT) via a system that can be implanted into an animal and release CORT at a controlled rate. Using rattlesnakes as a model animal, this project investigated the preparation and characterization of CORT-loaded ethylene vinyl acetate (EVA) microparticles to experimentally elevate CORT levels. The EVA/CORT microparticles were prepared by an emulsification-solvent evaporation technique and characterized via FTIR, SEM, and TGA. The release of CORT from the microparticles was investigated *in vitro* at 25 °C in a reptile Ringer's solution over a period of five days at one day intervals. The CORT released was evaluated using a TGA analysis approach by analyzing the percent weight loss of CORT after each day. The prepared microparticles were found to have a wide range of morphologies and exhibited irregular shapes. Rather than displaying a distinct weight loss corresponding to CORT, the TGA data showed delayed degradation temperatures of the EVA/CORT microparticles as well as larger weight loss percents that corresponded to the acetic acid content of the EVA. These findings do not support the mechanism of CORT release from the microparticles. Therefore it can not be concluded that an EVA/CORT microparticle system was successfully prepared.

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List of Abbreviations

CORT	**CORT**icosterone
EVA	**E**thylene-*co*-**V**inyl **A**cetate
EVA/CORT-1	**EVA/CORT** microparticles batch **1**
EVA/CORT-2	**EVA/CORT** microparticles batch **2**
FTIR	**F**ourier **T**ransform **I**nfra**R**ed Spectroscopy
In vitro	In glass
Na-CMC	Sodium **C**arboxy**M**ethyl**C**ellulose
PE	**P**oly**E**thylene
SEM	**S**canning **E**lectron **M**icroscopy
TGA	**T**hermo**G**ravimetric **A**nalysis
VA	**V**inyl **A**cetate
wt%	**W**eigh**t** percent

Dedicated to my father, Eric, and my brother, Brad–the only two individuals who can keep me wild without leaving me rattled.

Chapter 1

Introduction

1.1 Background

For the past few decades, a popular trend in ecology and evolution has been the emergence of conservation physiology into its own discipline. Conservation science has continually been a vital force for ensuring biodiversity and sustainability, however it has not been until recently that researchers began to use physiological methods to gather data on pressing conservation problems. An important example is the discovery of the effects of dichloro-diphenyl-trichloroethane (DDT) on the reproductive behavior of predators as outlined by Wikelski and Cooke. When the connection was made between DDT and the failure of reproduction in many exposed predators, the use of DDT in substances was banned in major parts of the world. This DDT tragedy supports the potential insights that physiological studies of animals can reveal. They can determine cause and effect relationships that are important for conservation and even monitor the effectiveness of current conservation management methods (Wikelski and Cooke, 2006).

In this spirit, conservation physiology can assess the increasing influence humans have on natural systems. To supply for our own needs and expansion, humans have been known to induce habitat destruction, overexploitation of resources, and biological exploitation. In turn, these human activities decrease the number of suitable habitats for wildlife (Dickens and Romero, 2013). These events pose serious threats to disrupting the life-history stage of animals by inducing a stress response that animals have not adapted to handle. The key component to study these threats is the physiological response of stress in animals, i.e. the fluctuation of hormones in animals resulting from such events. By studying these responses in great deal, conservation biologists can provide an assessment of the consequences that human influence has on species reproduction rates and population numbers, two key metrics of biodiversity.

1.2 Review of Literature

1.2.1 Stress in Organisms

The definition for the state of stress that animals experience due to external factors is typically debated due to the wide variability in historical experimental methodologies. Nonetheless, it has been generally accepted that, broadly, stress is a state an organism experiences when they are faced to cope with unpredictable and adverse conditions (Claunch et al., 2017). Coping with such capricious conditions is not typically part of the life history cycle of animals and thus are not adapted to handle such conditions.

These conditions are therefore a source of perturbation for animals. Factors that can induce such perturbation can include those from physical environments (e.g. storms, climate change, etc.), changes in the number of major predators, and changes that alter social status which consequently reduces the access to resources (Wingfield, 2005).

Augmented with stressors that result from human influence, these perturbation factors likely become intensified when inflicted on the animal. Even more so, if perturbation is prolonged or frequent, then there is a higher potential for severe stress. However, all organisms must be able to cope with any unpredictable event at some stage in their life history cycle (Wingfield, 2005). Although, it has been theorized that conditions that induce chronic stress in many wild organisms are expected to increase over the next coming decades (Wingfield, 2005). This is the result of many factors such as climate change and the increasing influence of humans on natural habitats. Depending on the event, the stress response experienced can be divided into two categories: acute, short-term and chronic, long-term.

Acute, short-term responses are typically referred to as a collection of physiological and behavioral changes that are thought to help an animal survive in the wild (Sapolsky, Romero, and Munck, 2000). The role of such changes is similar to the process of allostasis. The purpose is to temporarily stop normal life-history functions in order to quickly counteract the impact the stressor had on the animal and allow the animal to return back to its normal activities (i.e. homeostasis) (Dickens and Romero, 2013). Chronic, long-term responses occur when the stress response system of an animal has been pushed far beyond its normal capacity. This can be the result from either fluctuating intensity of a stressor and/or persistence of the stressor. Typically, chronic, long-term responses have a major impact on the health of the animal, however it is worth noting that there is variation in the effects of both responses based on the magnitude of the response (Dickens and Romero, 2013).

1.2.2 Role of Corticosterone

Paramount to understanding the stress response of animals is the physiological reaction once a stressor is encountered. To cope with the capricious conditions, organisms typically respond by elevating levels of circulating glucocorticoids (GCs), such as coritsol and/or corticosterone (CORT) (Claunch et al., 2017). It is believed that these hormones typically help mediate any ongoing or pending stress response via elevation permitting other features of the stress response to emerge, and/or the levels actively inducing the stress response (Sapolsky, Romero, and Munck, 2000). Although, severe elevation of these hormones can have negative effects on animals. Studies has shown that the severe, chronic stress response can influence the expression of a suite of traits in many taxa, including reproduction, immune system function, behavior, thermoregulation, and protein metabolism (Claunch et al., 2017; Wingfield et al., 1998; Martin, 2009; Landys, Ramenofsky, and Wingfield, 2006; Wingfield and Sapolsky, 2003).

As a result of studies on such chronic stress responses, physiological stress responses and baseline CORT levels are often used to assess the health of many populations of animals in the wild. This is so because CORT and other glucocorticoids can be correlated with both environmental chance and fitness parameters (Busch and Hayward, 2009). Additionally, such research can be used to predict future growth and reproduction, insights that are continually valuable for conversation science. It has become apparent in

recent literature that such physiological studies in ectotherms (such as rattlesnakes) can yield interesting insights into the effects of elevated CORT levels since because of their slow metabolism, they can endure chronic CORT elevation for longer periods of time (Landys, Ramenofsky, and Wingfield, 2006; Claunch et al., 2017).

1.2.3 Challenges with Corticosterone

The key to test hypotheses about effects of chronic stress responses is the ability to experimentally manipulate CORT levels. Rather than exposing organisms to acute stressors to stimulate a chronic stress response, sustained CORT levels via direct experimental manipulation has been theorized to better mimic CORT levels during chronic stress (Claunch et al., 2017). Experimental studies of this sort are rare partially due to difficulties in experimentally manipulating elevated CORT levels, such as repeated application of a stimulus to attain CORT concentration (Sopinka et al., 2015).

There have recently been several studies that attempted to evaluate the effects of elevated CORT levels in wild populations. French et al. reported manipulated CORT concentrations in female tree lizards to test the role CORT plays in the regulation of limiting resources between reproductive and immune systems (French et al., 2007). To experimentally manipulate CORT levels, an implantable gelling material was used that allowed slow release of the hormone. The implant contained poly(ethylene gylcol)diacrylate mixed with CORT in liquid form which after injection experiences a gelling reaction that releases the CORT (French et al., 2007). Though effective at elevating CORT concentrations, the gelling material provided the elevation due to an initial pulsatile release of CORT soon after injection. In contrast, to evaluate the effects of elevated CORT levels, it is important to provide a means of controlled release of CORT for a prolonged period of time that allows the researcher to aptly measure changes in behavioral and/or reproduction traits.

A study by Claunch et al. reported the use of an implant as well to experimentally manipulate CORT levels in a wild population of snakes. The implant consisted of silastic tubing infused with crystalline CORT which would allow for controlled release of CORT throughout the ends of the cylindrical implant. Moderate sustained release of CORT provided a basis for the determination of behavioral changes in rattlesnakes due to chronic CORT elevation. However, the results suggest that rattlesnakes do not respond to chronic CORT elevation the same way as other organisms (Claunch et al., 2017). The intentions of this study are to similarly experimentally elevate CORT levels in rattlesnakes via a controlled release system that maintains CORT levels at longer periods of time.

1.2.4 Polymers for Drug Delivery

To study the effects of elevated hormone levels in animals, attention has been brought to mechanisms in which hormone levels can be altered in humans. This functionality is often regarded in science and engineering as drug delivery, where devices and systems are designed to control the release of a drug. In this context, "drug release" refers to the process in which drug solutes migrate from initial positions in the system to the outer surface and then to the release medium (Langer, 1990). Though seemingly straightforward, this process is affected by multiple variables such as the physiochemical properties of the drug, the structural characteristics of the material system, and

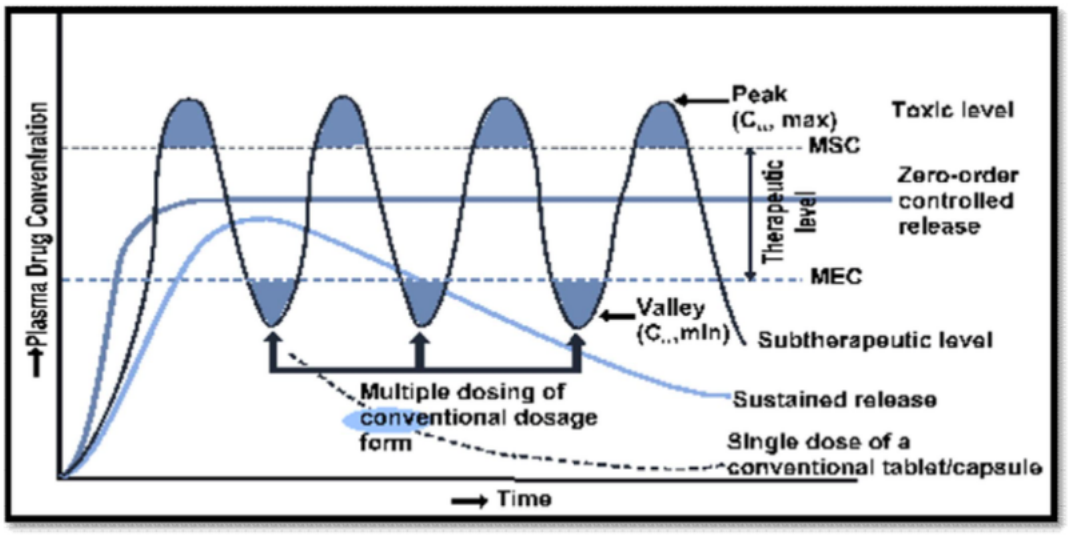


FIGURE 1.1: Hypothetical plasma drug concentration profiles of multiple doses of conventional drug forms and single doses of sustained release and controlled release formulations. Adapted from Misal et al.

possible interactions between these factors (Fu and Kao, 2010). It is of continual interest to researchers to select materials considering these driving factors and the specific purpose of the system.

Typically, drugs follow a peak and valley release profile as depicted in Figure 1.1. After administration, the drug level increases to a desired therapeutic level slowly. Then due to variable conditions inside the body, the concentration of the drug continues to increase past this desired level and then decreases slowly after a critical point.This therefore results in fluctuations of the plasma drug level between when it is ineffective or possibly toxic (Misal et al., 2013). Similarly, many tablet formulations have been designed to produced a "sustained" release of the drug where the plasma drug concentration remains in the therapeutic level longer before coming back down (Figure 1.1). To solve this problem, mechanisms have been designed to produce drug release profiles where the drug concentration reaches the desired therapeutic level inside the body and stays there for a desired period of time. This zero-order release kinetics behavior is commonly referred to by researchers as controlled release. Depending on the polymer and drug, such systems can be designed to maintain consistent drug levels from hours to years (Langer, 1990).

Both non-degradable and degradable polymers have dominated as the primary materials of interest for controlled drug delivery in literature. Generally, the mechanisms of release from these polymers can be classified as diffusion driven, chemically-controlled, or solvent-controlled. In the case of diffusion driven systems, Fick's law of diffusion provides a mathematical model for the transport of drugs through polymeric matrices. It is described by Grassi and Grassi that the model of Fick's law refers to the transport of the drugs when the polymer relaxation time is much greater than the solvent diffusion time (Grassi and Grassi, 2005). Non-degradable polymers are typically used in such diffusion-controlled systems in the form of a matrix or membrane reservoir system (Fu and Kao, 2010). In chemically-controlled systems, drugs can be attached to

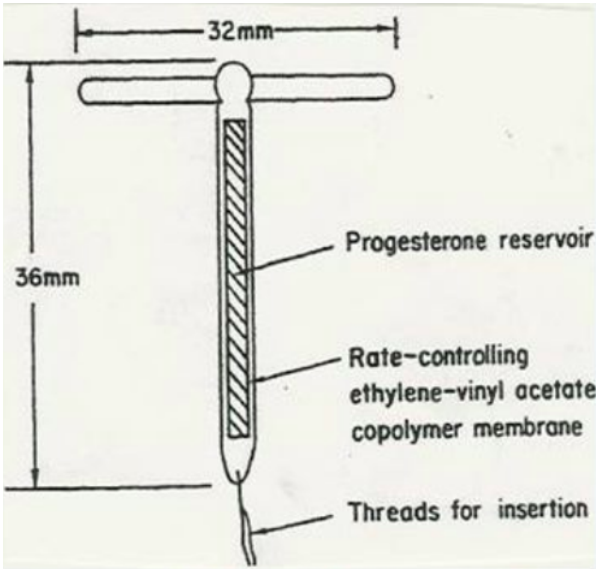


FIGURE 1.2: Diagram of the Progestasert system. Adapted from Salem & Baskin.

the polymer via pendant chain chemistry where the drugs are cleaved by water which initiates release of the drug (Langer, 1990). Because of the required influx of water, degradable polymers comprise the majority of these systems. Solvent controlled systems are based on the influx of a solvent (i.e. water) that causes the polymer to swell which creates a medium for the drug to be released into the surrounding environment (Langer, 1980). Like chemical-controlled systems, solvent-controlled systems also typically use degradable polymers activate the swelling mechanism of release. A majority of the aforementioned systems typically control the release of drugs on the order of a few days. To study behavioral traits of ectotherms, drug delivery systems would have to be modeled to release hormones on the order of thirty days or longer. Perhaps the most popular use of controlled release systems that sustain drug levels this long (years) are contraceptive implants. The Progestasert system shown in Figure 1.2, for example, controls the delivery of the hormone progesterone for one year from an intrauterine implant (Salem and Baskin, 1992). Using implants of this sort, the level of the hormone is kept at a constant level where treatment becomes effective without inducing any side effects for the patient. Even more so, because of the long life of the implant, patient compliance is less of a concern since there is no requirement of consistent administration of the hormone. Since the development of the Progestasert system, many other contraceptive implants have been developed that have the same functionality, such as Implanon and Nexaplanon (Schneider et al., 2017).

Contraceptive implants of this sort are based on a membrane reservoir system using a non-biodegradable polymer. Reservoir-type devices refer to those that have an inert coating material around a matrix of the the same coating material and the drug ((Fung and Saltzman, 1997), Figure 1.3). The coating material functions as a rate-controlling membrane that allows for zero-order release of the drug over time. This release rate remains relatively constant and is not affected by concentration, but is more related to physical parameters, such as the membrane thickness and surface area of the device,

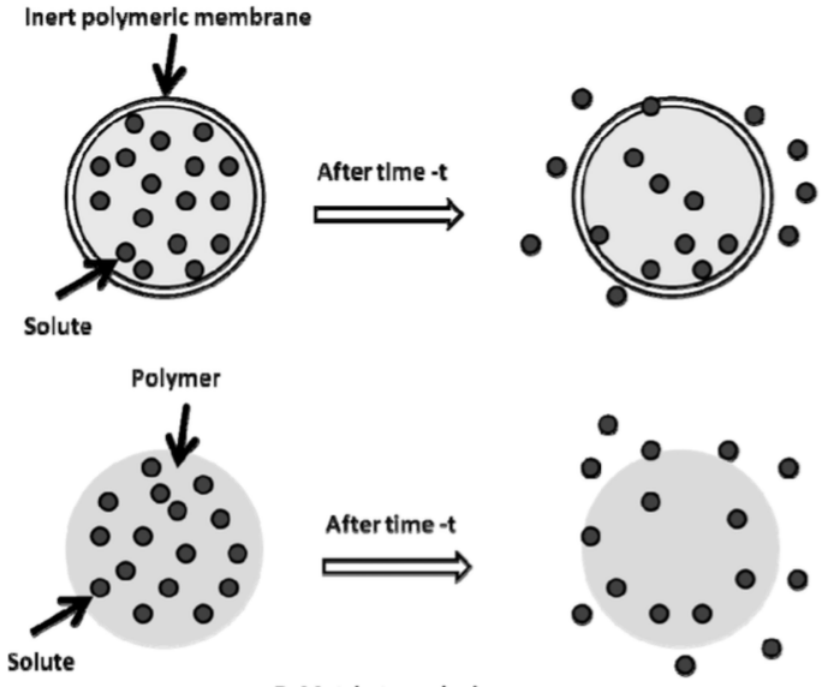


FIGURE 1.3: Schematic representation of membrane-reservoir controlled release systems and matrix controlled release systems, respectively. Adapted from Fung & Saltzman.

and permeability of the membrane (Langer, 1980). In contrast, matrix-only type devices tend to be Fickian diffusion driven, which is affected by the concentration gradient, diffusion distance, and degree of swelling which all can impact the final drug release profile of the system (Figure 1.3) (Fu and Kao, 2010).

1.2.5 Ethylene Vinyl Acetate for Controlled Release Systems

A majority of membrane reservoir delivery systems have been fabricated using ethylene vinyl acetate (EVA) copolymer as the material for the matrix as well as the inert membrane. EVA is a transparent thermoplastic copolymer of ethylene and vinyl acetate (VA) monomers in which the VA segments are randomly distributed throughout the ethylene polymer backbone. The VA content in commonly available EVA ranges between 1 and 40 wt.%. Typically, many chemical and mechanical properties arise from the VA content such as crystallinity, melting point, stiffness, and even polarity (Schneider et al., 2017). An important property relevant to membrane systems that arises from VA content is the permeability of the copolymer. As the VA content increases, the overall rigidity of the copolymer chain decreases and consequently the polymer becomes more rubbery and permeable therefore allowing a greater drug release rate to be observed (Tallury, Alimohammadi, and Kalachandra, 2007).

In the pharmaceutical industry, EVA is most commonly processed by extrusion (Schneider et al., 2017). The extrusion process, usually involving a hot melt, is advantageous for producing systems that depend on large surface area to volume ratios. Though, other techniques have been reported to process EVA for applications including but not

limited to controlled-release systems. A report by Kalachandra, Dongming, and Offenbacher reported a solvent casting technique for the production of EVA thin film drug delivery devices. The methodology uses dichloromethane and 40 wt% VA content EVA to prepare the polymer thin films that encased tetracycline, minocycline, or nystatin (Kalachandra, Dongming, and Offenbacher, 2002). Other processing techniques include injection moulding, dip coating, spray coating, sintering, and coacervation (Schneider et al., 2017). One of the oldest and perhaps most industrially feasbile techniques of coacervation has been microencapsulation by emulsification solvent evaporation, which is the methodology of interest in this study.

1.2.6 Microencapsulation By Emulsification Solvent Evaporation

The procedure of microencapsulation by emulsification solvent evaporation is conceptually straightforward. First, it involves the emulsification of a polymer solution-containing drug into a second, liquid phase containing an emulsifier to form a dispersion of drug-polymer solvent droplets (Watts, Davies, and Melia, 1990). Second, the solvent is removed from the droplets by evaporation leaving a suspension of drug-containing polymer microspheres. The microspheres can then be separated, washed, and dried. This technique can be tailored to produce microspheres of a wide size range, from 200 nm to several hundred microns where the structure of the microspheres is continually the drug dispersed through a matrix as a solid or a molecular dispersion (Watts, Davies, and Melia, 1990).

The role of the emulsifier in the microencapsulation is the short-term stabilization of suspended polymer droplets. This stabilization process prevent concomitantly prevents aggregation and coalesence of the polymer (Watts, Davies, and Melia, 1990). According to Watts, Davies, & Melia, this sequence of events is potentially short since after evaporation occurs and the polymer droplets begin to harden, aggregation and coalesence is not expected to occur. Most commonly used emulsifiers include polymeric stabilizers such as gelatin, PVA, and sodium methylcellulose. These stabilizers are popular due to the potential increase in solution viscosity these can produce (Watts, Davies, and Melia, 1990). Higher solutions viscosities have been reported in literature to produce distorted microspheres of varying shapes (Cavalier, Benoit, and Thies, 1986).

A critical aspect to the microencapsulation procedure is the selection of a solvent for the emulsification. Important criteria for selecting a solvent are the ability to dissolve the EVA polymer and the drug, immiscibility with the emulsifier, lower boiling point, and low reported toxicity (Watts, Davies, and Melia, 1990). An interesting study reported by Chowdary & Babu investigated the surface effects and permeability of the resulting microspheres by using different solvents. The various solvents tested were cyclohexane, dichloromethane, 1,2-dichloroethane, and chloroform using a similar technique to that explained above. High permeability and drug release was observed in the case of microspheres prepared using as the solvent for the polymer EVA (Chowdary and Babu, 2003). The order of increasing permeability of the microspheres employing the various solvents was cyclohexane, dichloromethane, 1,2-dichloroethane, and chloroform. It is believed that the solvent used induces nano-sized surface effects on the microspheres which thus increase or decrease the permeability of the EVA depending on the solvent.

The efficacy of microspheres produced by the emulsification solvent evaporation method has been well studied using a variety of different solvents and drugs in literature. For example, Chowdary, Enturi & Jagadish reported the use of EVA microcapsules to control the release of pioglitazone, an anti-diabetic medication. The emulsification technique utilized sodium carboxy methyl cellulose as the emulsifier and EVA dissolved in chloroform to form the homogenous polymer solution (Chowdary, Enturi, and Jagadish, 2011). It is interesting to note that the authors reported that both zero-order and first-order kinetic models are equally appliable to describe the release data. This was largely determined by an analysis of correlation coefficients and analyzing the data to an equation that yield different modes of transport. This equation is known as the Ritger-Peppas equation and is described as

$$\frac{M_t}{M_\infty} = k_1 t^n \tag{1.1}$$

where M_t is the amount of solute transported at time t, k is a constant incorporating characteristics of the particle system that makes up the formulation, and n is a determined exponent where n=1 corresponds to zero-order release, n=0.5 corresponds to Fickian diffusion, and where $0.5 < \text{n} < 1$corresponds to non-Fickian diffusion (Ritger and Peppas, 1987).

1.3 Proposed Research

The research being proposed in this study combines both conservation physiology and materials engineering. It is our goal to create an implant similar to past studies (Claunch et al., 2017; French et al., 2007) that can elevate CORT levels in rattlesnakes for a prolonged period of time. It is critical to maintain consistent CORT levels over the course of the thirty day period to allow accurate determination of effected behavioral traits as well as biological traits. We plan to achieve this using an EVA polymer device that encacpsulates the CORT within its core, which would allow controlled released via diffusion out of the EVA, thus yielding a zero-order release profile.

We anticipate using an emulsification-solvent evaporation technique to produce the EVA/CORT system. The result will be microparticles that encapsulate the CORT within the particle core at potentially different concentrations. Here, we describe the system as 'microparticles' because different analytical chemistry techniques would be required to determine the exact formulation of the microparticle (i.e. if it is a micro*sphere*, a monolithic system of EVA and CORT, or if it is a micro*capsule*). Figure 1.4 shows schematically the difference between these formulations. In the absence of determining which exact formulation is produced, the system will be referred to as microparticles.

Electron microscopy will be utilized to investigate the morphology of the resultant microparticles. Infrared spectroscopy will be used to examine details of the chemical structure of the microparticles after processing to determine any effects of processing on the chemistry of the materials. Testing of the viability of the microparticles to control the release of CORT will be done *in vitro* in a reptile Ringer's solution using thermogravimetric analysis (TGA) as an indirect measure of CORT release. Once the release profile has been determined, the system can be evaluated on its efficacy of releasing CORT at a controlled rate in order to maintain CORT elevation. Data from this study can help yield valuable insights into how human interactions continually affect the stress response in

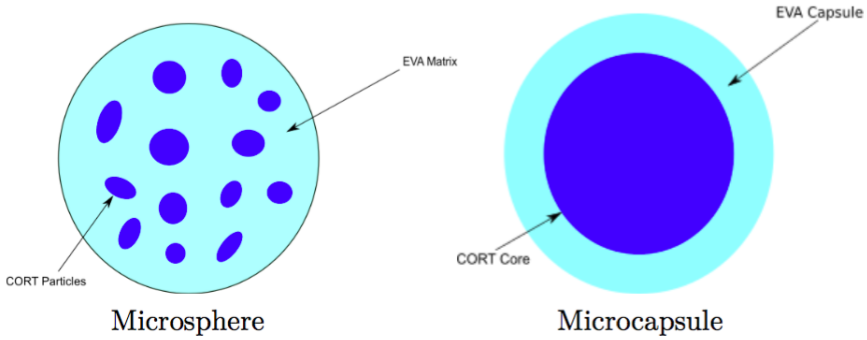


FIGURE 1.4: Schematic comparison of the two different formulations of microparticles that can be produced from the emulsification-solvent evaporation technique.

organisms and even more so how detrimental that response can be to a snakes', and reptiles' in general, life history cycle.

Chapter 2

Materials and Methods

2.1 Materials

The materials used in this study include ethylene-*co*-vinyl acetate (EVA), 12% VA content, crystalline corticosterone (CORT), cyclohexane (anhydrous) as the organic solvent to dissolve the EVA, 0.5 wt% sodium carboxymethyl cellulose (Na-CMC) as the emulsifier of the microparticles, and a reptile Ringer's solution composed of various proportions of salts listed below in Table 2.1. All chemicals were used as-received.

TABLE 2.1: Components of the Reptile Ringer's Solution

Component	Amount
KCl	0.35 g
KH_2PO_4	0.1625 g
$MgSO_4$	0.29375 g
$NaHCO_3$	1.68 g
NaCl	8 g
$CaCl_2$	0.2775 g
DI Water	1 L

2.2 Preparation of EVA/CORT Microparticles

2.2.1 Initial Process

The method used in this study to prepare the EVA/CORT microparticles was a technique called emulsification-solvent evaporation. In this process, an emulsifier is used to precipitate a solution of the copolymer and drug as a fine dispersion of droplets, where following this step, the solvent used to dissolve the polymer is evaporated leaving behind only the polymer and drug encapsulated in microparticles. The exact procedure is adapted from previous studies producing EVA microspheres and microcapsules (Chowdary and Babu, 2001; Chowdary, Enturi, and Jagadish, 2011; Tallury, Alimohammadi, and Kalachandra, 2007; Chowdary and Babu, 2003; Watts, Davies, and Melia, 1990).

The effectiveness of the emulsification-solvent evaporation technique for producing microparticles was first tested by using EVA alone. First, a 1 wt% solution of Na-CMC was created by dissolving 2 g of Na-CMC powder in 200 mL DI water using a Premier Mill Corp. Series 2000 Laboratory Dispersator overhead mixer at approximately 1000

RPM. 500 mg of EVA copolymer was then dissolved in 25 mL of cyclohexane at 30 °C while magnetic stirring in order to form a homogenous polymer solution. Once a homogenous polymer solution was obtained, 10 mL of the polymer solution was added in a thin stream to the Na-CMC solution while continually stirring at approximately 1000 RPM to emulsify the added solution as fine droplets. The solvent, cyclohexane, was then removed by continuous stirring at room temperature for 3 h.

After the solvent was allowed to evaporate for 3 h, the microparticles were collected by gravimetric filtration. Using a ceramic funnel and filter paper with a pore size of 20-25 microns the Na-CMC solution and microparticle precipitates were separated by filtering the Na-CMC solution overnight. After the microparticle precipitates were entirely collected, they were washed repeatedly with DI water.

2.2.2 Process Optimization

To optimize the emulsification process and produce more discrete microparticles, the conditions for creating the Na-CMC solution were altered, A 0.5 wt% solution of Na-CMC was used by dissolving 1 g of Na-CMC powder in 200 mL DI water using the overhead mixer at approximately 1000 RPM, similar conditions to that reported in literature (Chowdary and Babu, 2001; Chowdary and Babu, 2003; Chowdary, Enturi, and Jagadish, 2011). Because the Na-CMC absorbs water rapidly, the Na-CMC was added slowly in small amounts to the DI water in the mixer to prevent clumping of the Na-CMC powder. Furthermore, to facilitate the ease of collecting the microparticles, vacuum filtration was used in place of gravimetric filtration using a Pro-Set 1 stage vacuum pump.

2.2.3 Final Process

The final process included loading the microparticles with CORT. This was done similarly as already discussed by dissolving 500 mg of crystalline CORT in the polymer solution (500 mg of EVA dissolved in 25 mL cyclohexane). Then, 10 mL of the CORT+polymer solution was added in a thin stream to the Na-CMC solution under mixing conditions at approximately 1000 RPM. This step included different conditions regarding the location of where the thin stream was added in respect to the mixing paddle and the vortex. The solvent, cyclohexane, was then removed via evaporation by continuous stirring at room temperature for 3 h. After the solvent was allowed to evaporate for 3 h, the microparticles were collected by vacuum filtration and washed repeatedly with DI water. Once washed, the microparticles were allowed to dry overnight, where afterwards they were collected and stored at room temperature. The final emulsification-solvent evaporation technique is shown schematically below in Figure 2.1.

2.3 Characterization of EVA/CORT Microparticles

2.3.1 Determination of Functional Groups

To determine the functional groups, and in extension, to validate the chemical composition of the resultant microparticles, fourier transform infrared spectroscopy (FTIR) was

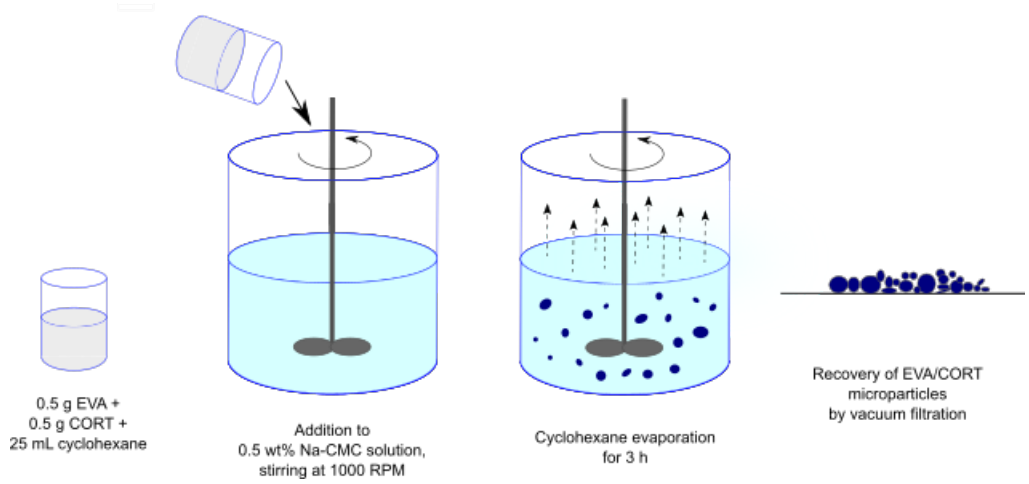


FIGURE 2.1: Final emulsification-solvent evaporation technique used in this study.

utilized. Using a Jasco FTIR 4600, spectra were taken from 4000 to 400 cm^{-1} at 2.0 cm^{-1} resolution of single EVA and EVA/CORT microparticles.

2.3.2 Morphology of EVA/CORT Microparticles

To characterize the morphology of the resultant microparticles, sample sizes representative of each batch were observed under a scanning electron microscope (SEM - FEI Quanta 200). The microparticles were mounted directly on to the SEM sample stub, using double sided sticking tape, and sputter coated with gold film under reduced pressure. Particle sizes were determined by using an estimation by scale bar pixel method of the obtained SEM images. This method consisted of determining the amount of pixels equivalent to the scale bar of the SEM image. Using that relationship, the diameter of the particles were determined by measuring the amount of pixels that made up the diameter of individual discrete particles.

2.4 *In vitro* Testing to Evaluate CORT Release

2.4.1 *In vitro* Method

To evaluate the release of CORT, the prepared EVA/CORT microparticles were tested *in vitro*. The following process is shown schematically in Figure 2.2. 13 mg of EVA/CORT microparticles from all batches were tested in 15 mL of reptile Ringer's solution for each day of study. The microparticles+Ringer's solution were placed in a 25°C water bath and shaker table for five days, with samples taken at one day intervals. The microparticles were recovered by the sample and separate method using filter paper, washed with DI water, and later stored at room temperature. The *in vitro* drug release experiments were run in duplicate for all batches.

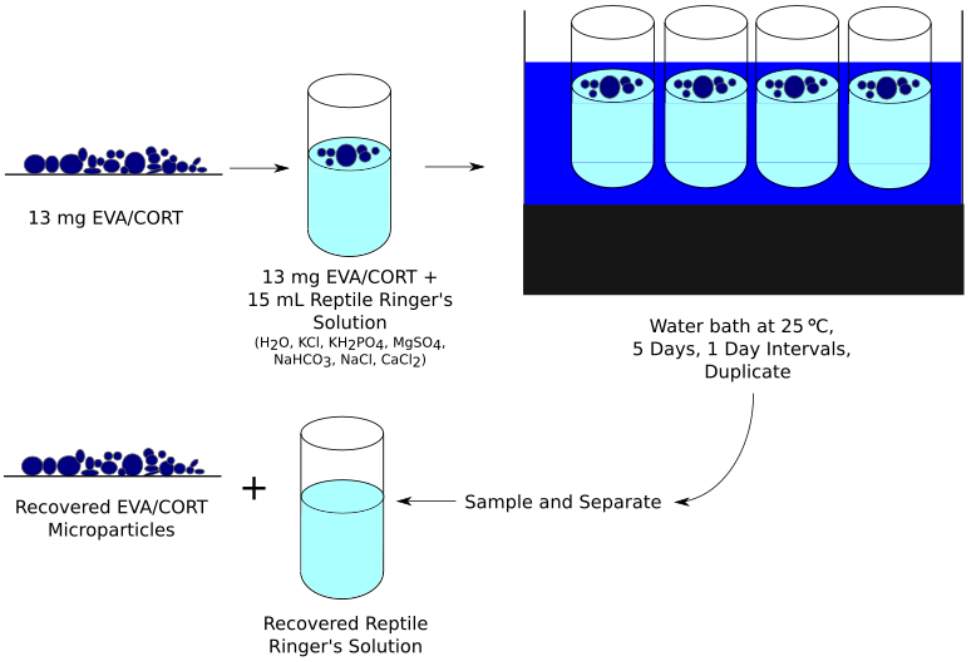


FIGURE 2.2: *In vitro* method used for testing CORT release.

2.4.2 Determination of CORT Release

To determine the action and amount of CORT release from the microparticles during *in vitro* testing, thermogravimetric analysis (TGA) was used as an indirect measure. After each day interval microparticles' were sampled and separated, the microparticles were run in a Mettler Toledo TGA/SDTA851 TGA at 10°C/min from 23°C to 500°C. All TGA data were converted to weight loss percent (wt%) in order to determine the weight loss of each component of the microparticles during *in vitro* testing. Consequently, this data would allow for an indirect method to construct a CORT release profile curve.

Chapter 3

Results and Discussion

3.1 FTIR Spectra

3.1.1 EVA Microparticles

The collected FTIR spectrum for the EVA microparticles alone is shown below in Figure 3.1. The spectrum appears to be representative of EVA due to the characteristic peaks for both PE and VA. The spectrum contains broad peaks at around 2900 cm^{-1}, 1420 cm^{-1}, and 720 cm^{-1}, all corresponding to different vibrational modes (stretch, bend, and rock, respectively) of the functional group methylene as reported in literature (D'Amelia et al., 2016). The spectrum also appears to have significant peaks at 1740 cm^{-1}, 1234 cm^{-1}, and 1015 cm^{-1}. These peaks are attributed to the bonding modes present in the VA, where 1740 cm^{-1} corresponds to C=O and 1234 cm^{-1} and 1015 cm^{-1} correspond to the C-O bonds branching off from the polyethylene backbone (Figure 3.2) (Wang, Liu, and Xiong, 2007; Meszlényi and Körtvélyessy, 1999).

The presence of these characteristic peaks in the EVA microparticles spectrum is evidence that there is EVA in the resultant microparticles. However, it is interesting to note the peak at around 2350 cm^{-1} that is typically not present in PE or VA spectra reported in literature. These two conditions leads to the speculation that there were possible effects of processing the as-received EVA into microparticle form.

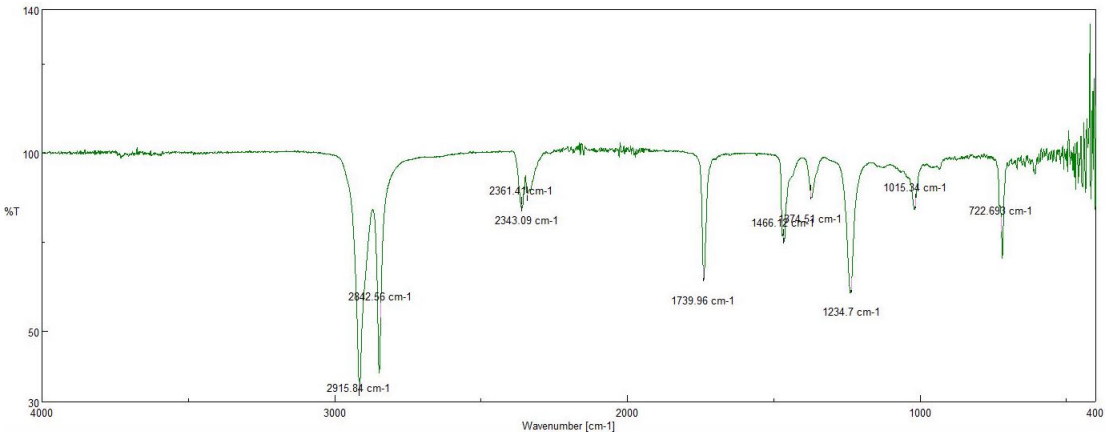


FIGURE 3.1: FTIR spectrum of EVA microparticles.

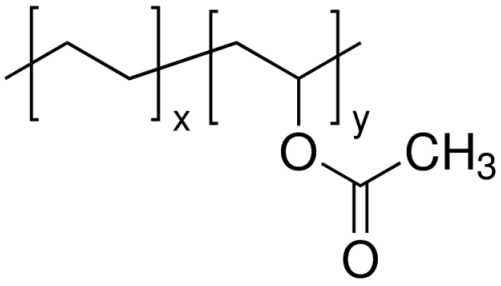


FIGURE 3.2: Chemical structure of EVA.

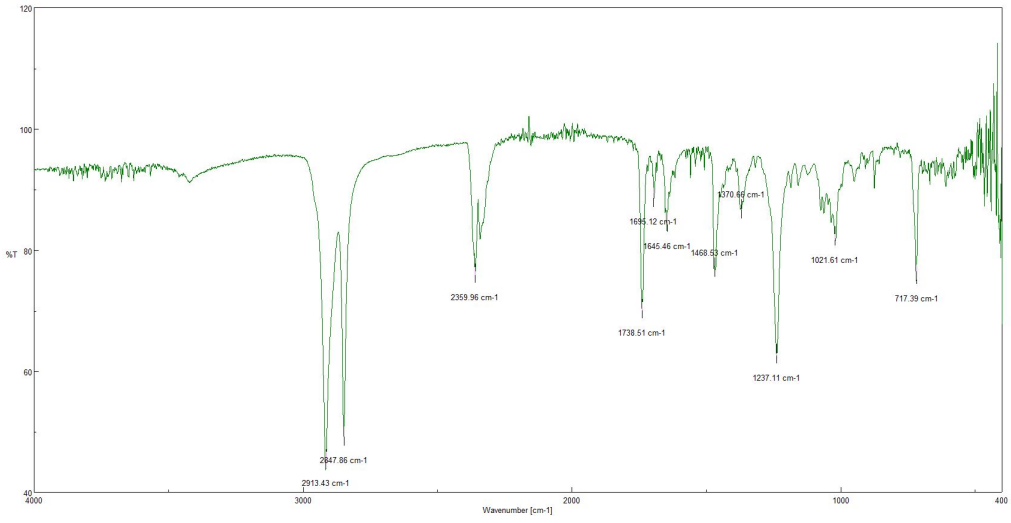


FIGURE 3.3: FTIR spectrum of EVA/CORT microparticles.

3.1.2 EVA/CORT Microparticles

The collected FTIR spectrum for the prepared EVA/CORT microparticles is shown in Figure 3.3. Like the spectrum from the EVA microparticles in Figure 3.1, the spectrum contains significant peaks at 2900 cm^{-1}, 1740 cm^{-1},1420 cm^{-1}, 1234 cm^{-1}, 1020 cm^{-1}, and 720 cm^{-1}. Using these observations in the data, it can be pointed out that these significant peaks are characteristic of the EVA in the prepared EVA/CORT microparticles.

The difference between the spectrum collected from the EVA microparticles and EVA/CORT microparticles is the presence of two moderate peaks at around 1650 and 1690 cm^{-1} in the EVA/CORT microparticles' spectrum.The potential of these peaks representing CORT in the prepared microparticles is a bit speculative due to the lack of infrared spectroscopy studies on CORT. However, there is one study by Shah, Ashraf-Khorassani & Taylor that reported the spectra of different steroids (including CORT) using supercritical-fluid chromatography (SFC)-FTIR (Shah, Ashraf-Khorassani, and Taylor, 1988). In the study, the authors reported the spectrum of CORT to have one large, broad peak at around 1700 cm^{-1} (Figure 3.4). The similarity in position of this reported peak and the two preaks found in the EVA/CORT microparticles spectrum gives insight to the potential presence of CORT in the microparticle system. However, without

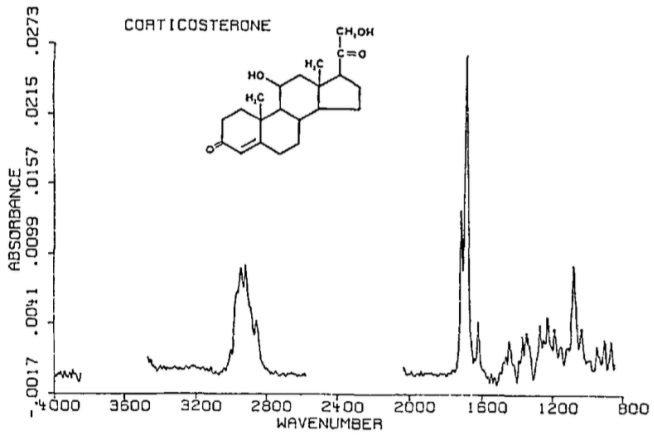


FIGURE 3.4: Published SFC-FTIR spectrum of CORT. Adapted from Shah, Ashraf-Khorassani & Taylor.

further infrared spectroscopic analysis into the origin of the peak (that reported in literature and those found in the EVA/CORT microparticles), the true presence of CORT can not be definitively determined. The presence of the peaks could be additive (where the EVA/CORT spectrum is simply an addition of the EVA spectrum and CORT spectrum) or it could be interactive, which would signify interaction between the EVA and CORT during processing.

Furthermore, it is interesting to note also the presence of the double peak at around 2340 cm^{-1} in Figure 3.3. The presence of this peak in the spectrum for the EVA/CORT microparticles as well points towards potential processing effects on the microparticles. There is a possibility that this peak corresponds to remaining traces of the solvent, cyclohexane, in the microparticle system. Such a finding is often the case with emulsification-solvent evaportation processes that utilize toxic solvents such as cyclohexane and dichloromethane (Watts, Davies, and Melia, 1990; O'Donnell and McGinity, 1997).

3.2 Location Results and Microparticle Yield

As discussed in Section 2.2.3, the final process for preparing the EVA/CORT microparticles utilized different conditions of where the stream of EVA+CORT was added to the emulsification. The different locations included directly above the mixer paddle, slightly further away from the mixer paddle, inside the vortex, and at the center of the vortex. Despite the success of the technique in producing EVA microparticles as just discussed, it was found that 50% (2 of the 4) of these conditions were unsuccessful in producing discrete microparticles. Instead, the EVA+CORT mixture appeared to clump up and agglomerate, thereby creating one large microparticle on the order of centimeters. A summary of these mixing conditions is displayed in Table 3.1. Because the theory for this system requires discrete microparticles, those batches that produced single, large microparticles (inside the vortex, and at the center of the vortex) were not used in the subsequent methods of this study. The batches that produced discrete microparticles (above the mixer paddle and slightly further away from the mixer paddle) were

used for the subsequent methods of this study and from on will be known as batch 1 (or EVA/CORT-1) and batch 2 (or EVA/CORT-2), respectively.

TABLE 3.1: Different mixing locations used and result in producing discrete microparticles.

Location	Microparticles?
Directly above mixer paddle	Yes
Inside the vortex	No
Center of the vortex	No
Further away from mixer paddle	Yes

It is believed that for the locations that caused the EVA+CORT to clump up, the shear force from the mixer paddle in the Na-CMC was not high enough in order to precipitate the solution as droplets. Conversely, for the locations that did produce discrete microparticles, the shear force was high enough, thus causing the stream to be sheared and precipitate droplets of the EVA+CORT solution. The total yield, by mass, of EVA/CORT-1 microparticles was 203 mg where the total yield of EVA/CORT-2 microparticles was 239 mg.

3.3 Morphology

3.3.1 EVA Microparticles

The morphology of all the microparticles produced in this study was analyzed using SEM. Figure 3.5 below displays an SEM image of a sample of EVA microparticles without CORT. Initially, it can be observed that a portion of the microparticles are not discrete. Some of the microparticles are not necessarily particles of EVA, but moreover, they are clusters of EVA. These few clusters of EVA are left out of the morphological analysis, due to the difficulty in using a standard method (by pixels of the SEM image) to evaluate their size.

Using the scale bar pixel method as described in Section 2.3.2, a summary of the morphology of the EVA microparticles is displayed graphically by a histogram in Figure 3.5. The size distribution was found to be relatively normal, with slightly more particles being on the larger end of the size distribution. The average diameter was found to be 831 microns. This particle size is similar to that reported in literature Chowdary and Babu, 2001; Chowdary and Babu, 2003; Chowdary, Enturi, and Jagadish, 2011, which thereby points to the success of the technique used in this study for producing microparticles.

3.3.2 EVA/CORT Microparticles

Figure 3.6 below shows an SEM image of a representative sample of the entire EVA/CORT-1 batch. Compared to EVA miroparticles, it can be initially pointed out that the EVA/CORT-1 microparticles have a more irregular shape, and yet still consist of various 'clusters' of material. Figure 3.6 also displays the size distribution histogram of this overall sample of EVA/CORT-1 microparticles. Unlike the EVA microparticles, these tend to be skewed towards smaller particles, with a majority of the particles having a diameter in the range

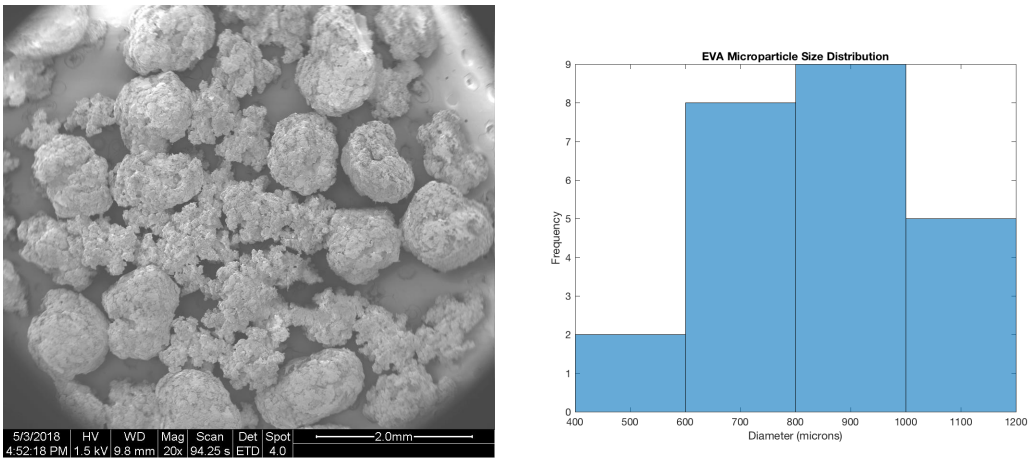


FIGURE 3.5: Left: SEM image of prepared EVA microparticles without CORT. Right: Size distribution histogram of EVA microparticles without CORT.

of 300-600 microns. The average diameter was found to be 734 microns, smaller than the EVA microparticles without CORT.

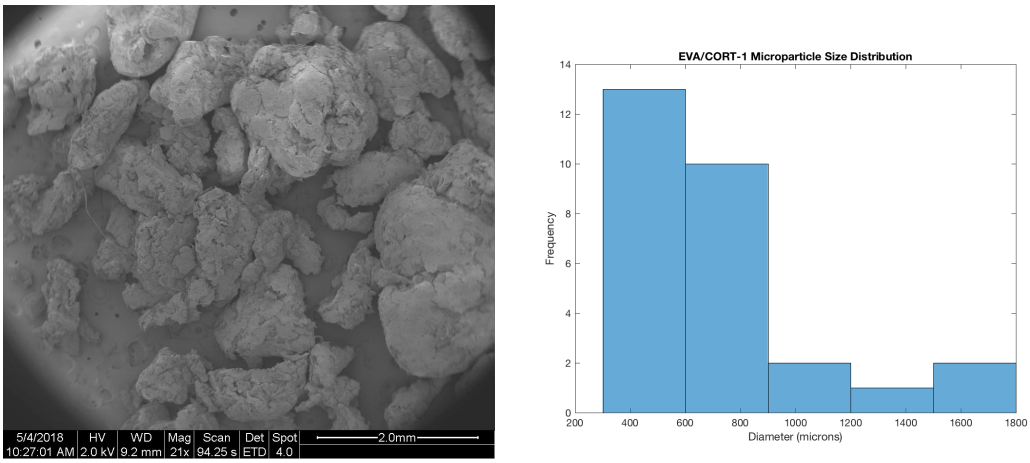


FIGURE 3.6: Left: SEM image of prepared EVA/CORT-1 microparticles. Right: Size distribution histogram of EVA/CORT-1 microparticles.

Comparatively, Figure 3.7 displays the SEM image and size distribution histogram of a representative sample of the entire EVA/CORT-2 batch. Like EVA/CORT-1 microparticles, the second batch contained microparticles that are irregular in shape, with various clusters dispersed throughout the sample. However, upon visual inspection, it appears that the EVA/CORT-2 microparticles are larger. The size distribution histogram shows that in this case, the EVA/CORT-2 microparticles have a more uniform size distribution, but with a majority of the particles having a diameter in a larger size range, 600-900 microns. Even more so, the average diameter was measured to be 886 microns, larger than the average diameter for the EVA/CORT-1 microparticles.

These morphological results point towards an interesting correlation between the location at which the EVA+CORT solution stream was added and the resultant size of

the microparticles. For the case of EVA/CORT-1, the EVA+CORT stream was added directly above the mixer paddle, where it is believed that the shear force is the greatest. Therefore, with a high shear force, the microparticles have a greater driving force to precipitate as small droplets. On the other hand, in the case of EVA/CORT-2, the EVA+CORT stream was added slightly further away from the mixer paddle (closer to the side of the beaker), where it is believed that the shear force is lower since the shear force drops radially with relation to the mixer paddle. So with a lower shear force, there is a lower driving force to precipitate the solution as smaller droplets, thus resulting in microparticles that are slightly larger in size along with a more normal size distribution.

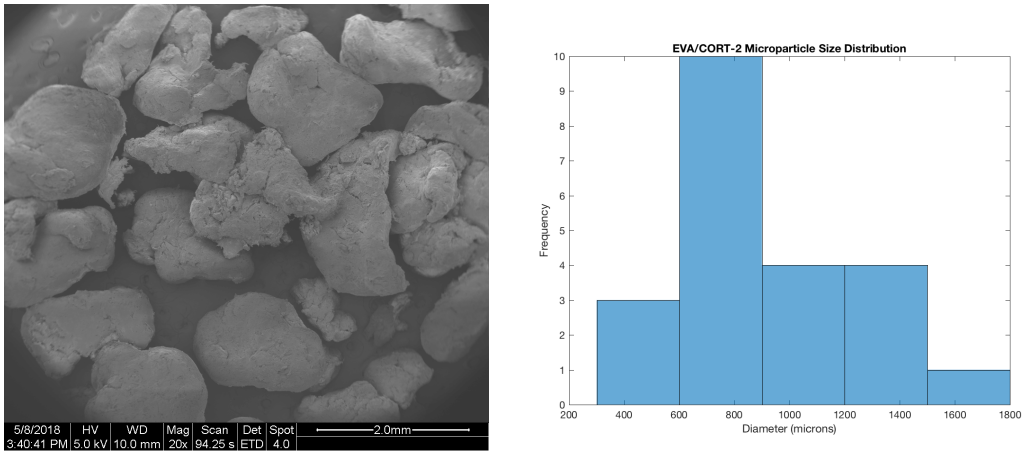


FIGURE 3.7: Left: SEM image of prepared EVA/CORT-2 microparticles. Right: Size distribution histogram of EVA/CORT-2 microparticles.

3.4 Surface Characteristics

3.4.1 EVA Microparticles

In addition to using SEM to evaluate the morphology of the prepared EVA/CORT microparticles, SEM was used to gather images of the surfaces characteristics of the microparticles. Figure 3.8 displays the SEM images collected of the surface of the EVA microparticles without CORT. As seen, the surface of the microparticles is not necessarily smooth. Moreover, the surface has a clustered feature to it, that appears to be composed of smaller clusters when seen at higher magnification.

3.4.2 EVA/CORT Microparticles

It is interesting to note that once the microparticles are prepared with CORT, the surface characteristics change drastically. Figure 3.9 and 3.10 show SEM images collected of the surface of EVA/CORT-1 and EVA/CORT-2 microparticles, respectively. Compared to the EVA microparticles without CORT, the surfaces of these microparticles appear more homogenous, i.e. they do not appear to have the clustered appearance that the EVA microparticles exhibited. This finding raises the thought that the EVA and CORT formed a solid solution under the emulsification process, thereby producing this homogenous

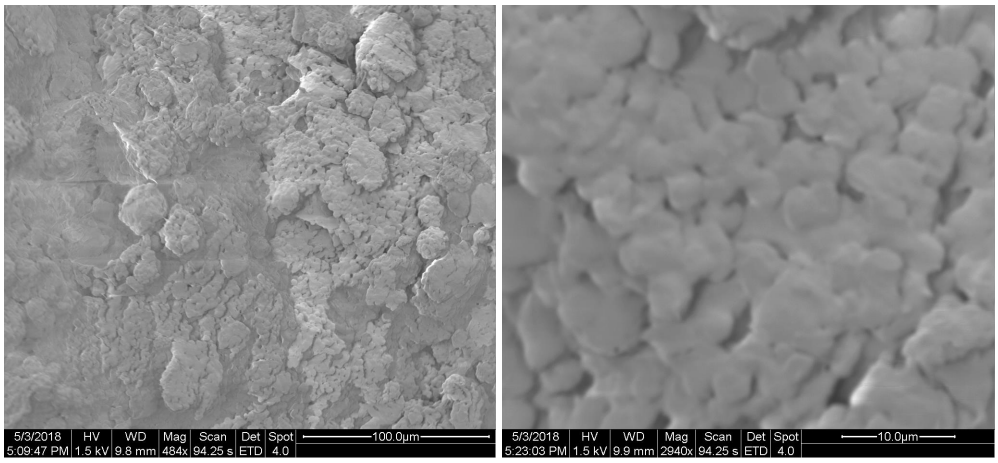


FIGURE 3.8: Left: surface of EVA microparticles without CORT. Right: surface of EVA microparticles without CORT at higher magnification.

surface. However, the SEM images also show that the microparticles' surfaces also appear to have pores and solid protrusions of material. These features are characteristic of the solvent evaporation aspect of the preparation technique since once the solvent evaporates, it either leaves behind a cavity in the material, or it causes the material to warp and concomitantly leaves behind various features such as pores (O'Donnell and McGinity, 1997; Chowdary and Babu, 2003). It has been determined in a past study that these features, such as pore size, have a direct effect on the drug release capability of the microparticle system (Chowdary and Babu, 2003).

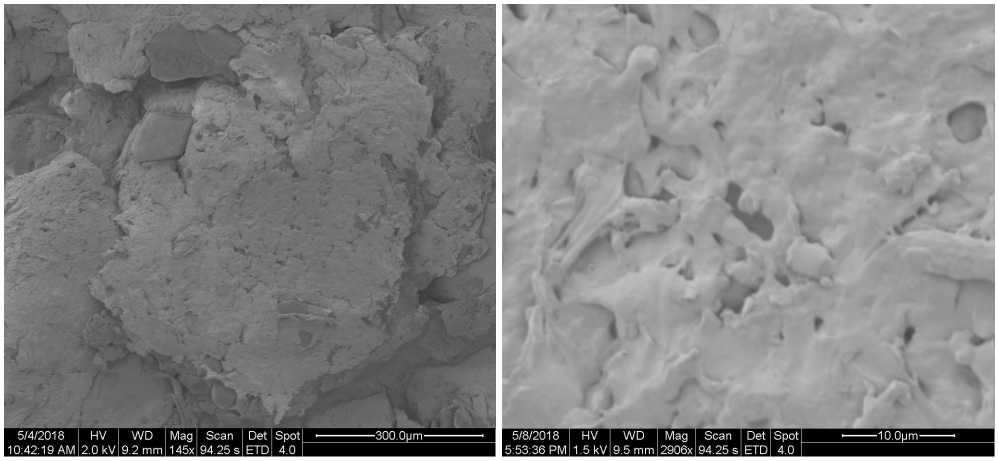


FIGURE 3.9: Left: surface of EVA/CORT-1 microparticles. Right: surface of EVA/CORT-1 microparticles at higher magnification.

3.5 *In vitro* Testing Results

To evaluate CORT release from the prepared EVA/CORT microparticles, thermogravimetric analysis (TGA) was used as an indirect measure. The theory behind using TGA as the method for evaluating CORT release is due to weight loss percent of different

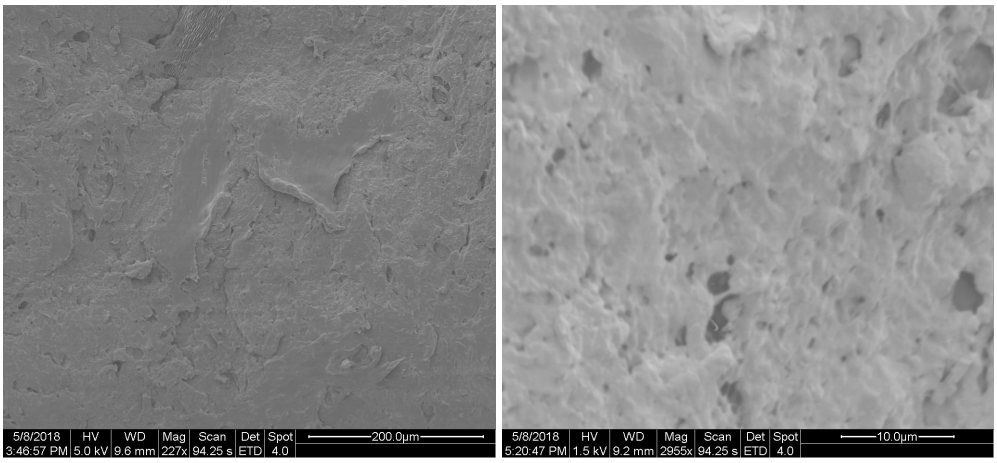


FIGURE 3.10: Left: surface of EVA/CORT-2 microparticles. Right: surface of EVA/CORT-2 microparticles at higher magnification.

components. Past studies in literature report that when TGA is run on EVA, the VA burns off first, then followed by the PE backbone (Williams, 1994; Wenwei et al., 1994). By analyzing the temperatures at which each component began and finished burning off, the total weight percent that component made up of the sample could then be determined.

Figure 3.11 below is a schematic (not data) that depicts the theory behind how such TGA data was to be used in this study. Since the EVA used in this study is composed of 12% VA, the TGA data from the EVA as-recieved should reflect a 12 wt% loss of VA, then followed by the remaining wt% loss of the PE (Figure 3.10a). Then, once the EVA/microparticles are prepared with CORT, the TGA data would reflect a new weight loss before the VA weight loss, which would thus correspond to the CORT being burnt off in the sample followed by the VA and PE loss (Figure 3.10b). Then, once the EVA/CORT microparticles have been recovered after each day of *in vitro* testing, the TGA data would reflect the same new weight loss, however in this case, the weight loss would be smaller than the initial loading and previous days, therefore pointing towards the action of CORT being released during the *in vtiro* experiment (Figure 3.10c). Using this data then, after each day interval of testing, a CORT release profile curve would be able to be constructed using the wt% loss (Figure 3.10d).

Despite the theory behind using TGA, the first interesting observation of the data is a consistent false reading of the weight loss in one of the EVA/CORT microparticles batches. In a majority of the samples from batch 2, after the samples were heated to above about 300°C, the weight loss reading dropped significantly to below 0 mg, then rose to a value above the starting weight, and oscillated back and forth like this over the course of about 75°C, and then dropped back to normal with a final weight loss of what appeared to be the PE in the sample (see appendix B for plots of this behavior). This observation happened in about 3 of the days and samples tested. Table 3.2 below gives a synopsis of the frequency of this false data occurrence. Because this behavior is starkly different from the analytical approach described in Figure 3.10, those days that exhibited this behavior could not be used to analyze any weight loss/release of CORT. Due to unfortunate logistical events, TGA data after day 4 was not collected, and therefore not included in this discussion.

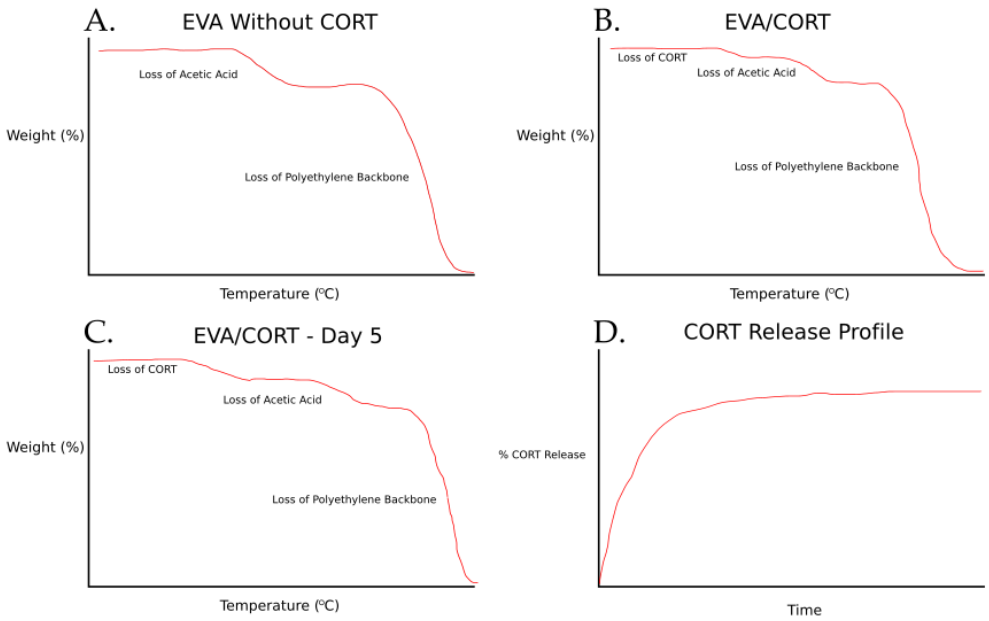


FIGURE 3.11: Theory of using TGA for evaluating CORT release indirectly. Schematic TGA plots of A) EVA without CORT, B) EVA/CORT microparticles at day 0 of *in vitro* testing, C) EVA/CORT microparticles after day 5 of *in vitro* testing, and D) cumulative CORT release profile curve generate using wt% loss after each day of *in vitro* testing.

TABLE 3.2: Frequency of aberration in TGA data for each batch and after each day of *in vitro* testing.

Batch	Day	Aberration?
1	0	No
2	0	Yes
1	1	No
2	1	Yes
1	2	Yes
2	2	Yes
1	3	No
2	3	Yes
1	5	No
2	5	No

The TGA curves for the EVA as-received and EVA microparticles without CORT are shown below in Figure 3.12. The two important, specific pieces of data in these curves are 1. the temperature at which the VA begins to burn off, and 2. the weight loss percent of the VA content. Figure 3.12a shows weight loss data of EVA as-received that is consistent with literature (Williams, 1994) where the degradation temperature is around 200 °C and the VA weight loss percent is 12%. Similarly, Figure 3.12b shows a similar degradation temperature around 200 °C, but reflects a weight loss percent of VA of 9.53%. The difference in weight loss percents points towards a speculation already discussed: potential effects of processing the EVA into microparticles on the

composition of EVA. Although, this is speculative since the difference in weight loss percent is not greatly significant.

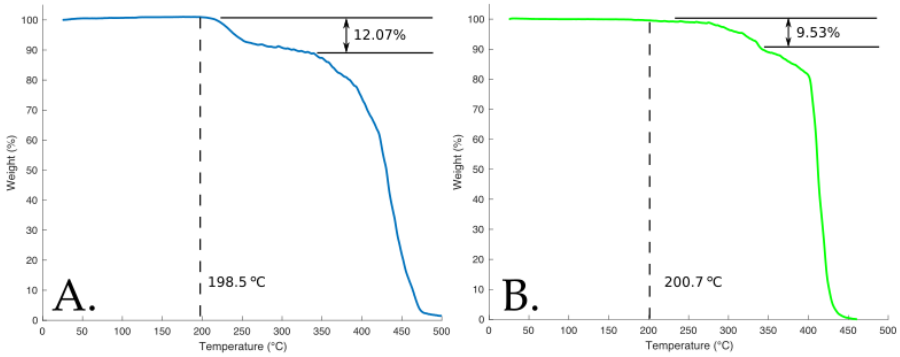


FIGURE 3.12: TGA curves of A) EVA as-received and B) EVA microparticles without CORT. Note the degradation temperatures and weight loss percents.

TGA curves for those days in which aberrations were not experienced by EVA/CORT-1 microparticles and EVA/CORT-2 microparticles are displayed in Figures 3.13 and 3.14, respectively. Initially, it is observed that the theorized first weight loss drop is not present in any of these data. The curves still have the overall shape of EVA alone. However, an analysis of these curves show two interesting trends. First, it was observed that as the days of *in vitro* testing progress, the degradation temperature increases as well, from 200 °C to around 250 °C. Second, it was observed that the weight loss percent increased, as the days of *in vitro* testing progressed. These trends are shown in Tables 3.3 and 3.4 for EVA/CORT-1 microparticles and EVA/CORT-2 microparticles, respectively.

TABLE 3.3: Summary of Degradation Temperatures and Weight Loss Percents for EVA/CORT-1 Microparticles

Day	Degradation Temperature (°C)	Weight Loss Percent
0	198.5 °C	12.82%
1	224.2 °C	16.12%
3	242.2 °C	16.71%
5	247 °C	18.99%

TABLE 3.4: Summary of Degradation Temperatures and Weight Loss Percents for EVA/CORT-2 Microparticles

Day	Degradation Temperature (°C)	Weight Loss Percent
0	235.8 °C	14.71%
5	251.8 °C	16.72%

Despite the absence of the first weight loss drop that would correspond to CORT, these findings bring attention to various experimental parameters that would effect CORT release. Since the degradation temperature of the EVA/CORT microparticles was found to be higher than that of the EVA as-received and EVA microparticles without

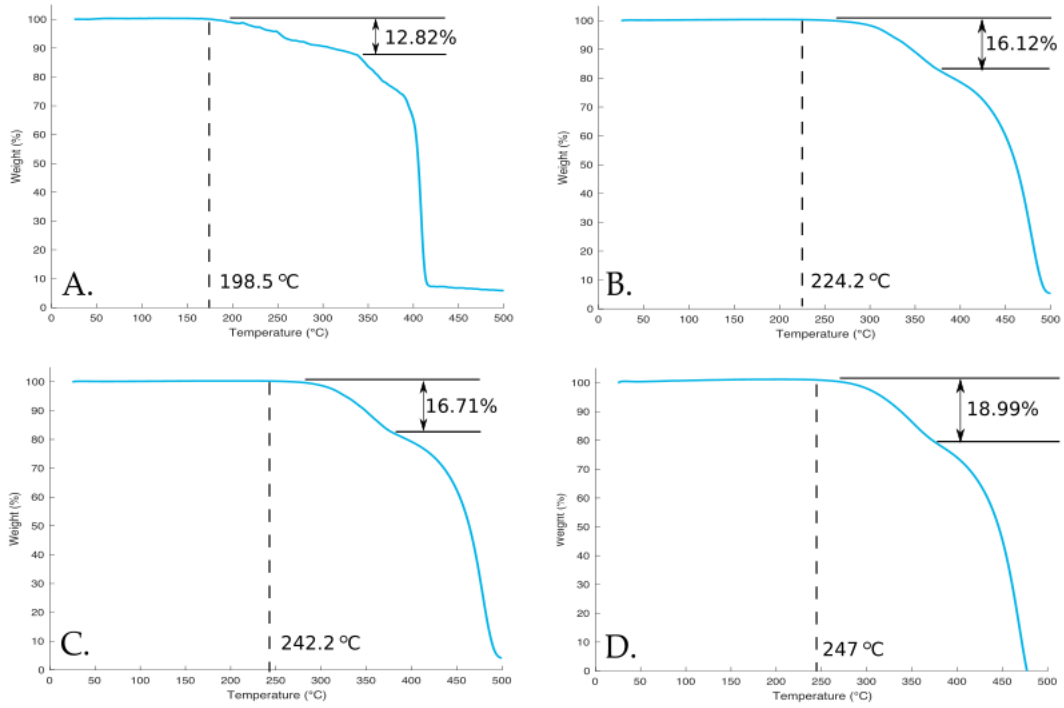


FIGURE 3.13: TGA curves of EVA/CORT-1 microparticles at A) day 0, B) day 1, C) day 3, and D) day 5 of *in vitro* testing. Note the degradation temperatures and weight loss percents.

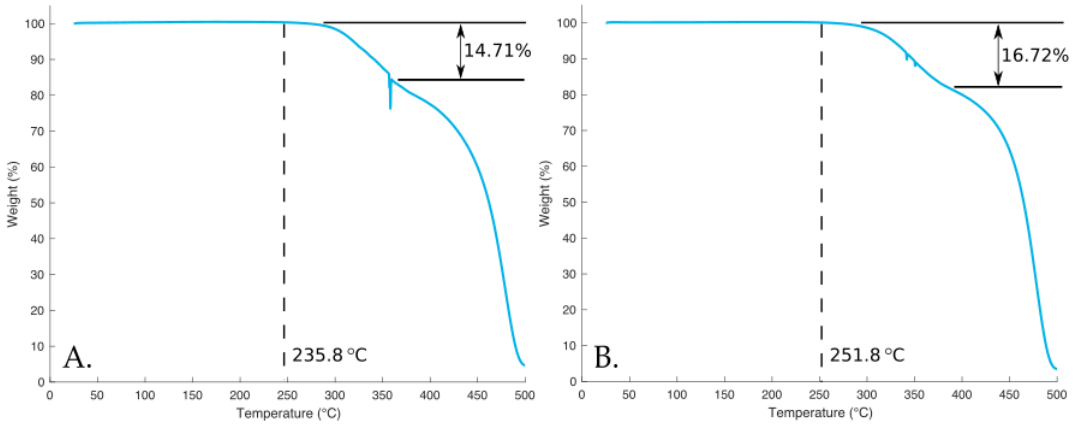


FIGURE 3.14: TGA curves of EVA/CORT-2 microparticles at A) day 0 and B) day 5 of *in vitro* testing. Note the degradation temperatures and weight loss percents.

CORT, it is interesting to speculate if the loading of CORT stabilized the microparticles to a greater extent. This stabilization could then have caused the VA content to burn off later, or it could have caused some solution of VA+CORT to burn off later if there was indeed interactions between the CORT and EVA.

In addition to the possibility of the EVA/CORT microparticles becoming stabilized when loaded with CORT, the increase in weight loss percent suggests an adverse effect

during the *in vitro* tests. Since the EVA contained 12 % VA content, it is counterintuitive that the weight loss percents are greater, and if the CORT release was part of the VA content drop, it would be understandable that the weight loss percent would decrease, however that was not the case. Considering these two details, the data suggests that there was some event during the *in vitro* tests that caused the microparticles to cumulatively gain weight over the course of the five days. It is a possibility that during the tests, a proportion of the Ringer's solution infiltrated the microparticles via the pores and/or cavities observed along the surfaces shown in Figures 3.9 and 3.10. This infiltration could then cause some of the salts to become entrapped in the microparticles which therefore would contribute to a greater weight loss in addition to the VA content. By this, the later days of the *in vitro* tests would yield higher weight loss percents than the earlier days, which was the case for both EVA/CORT-1 and EVA/CORT-2. Considering this finding, in addition to the increase in degradation temperatures, a CORT release profile curve could not be constructed.

Chapter 4

Conclusions

4.1 Preparation of EVA/CORT Microparticles

There exists a mismatch between the FTIR data of the EVA/CORT microparticles and the TGA curves after *in vitro* testing. The FTIR spectra suggests the possibility of CORT being encapsulated within the microparticles composed of EVA. However, the lack of a distinct weight loss drop in the TGA data suggests that the only components within the microparticles are VA and PE, the two components of EVA. Therefore, because of this mismatch, it can be concluded that microparticles were prepared in this study. However, the presence of CORT within these microparticles could not be identified.

4.2 Evaluation of CORT Release

The evaluation of CORT release depended only on the TGA tests done on the microparticles after each day interval of *in vitro* testing. Since there were was not a distinct weight loss drop in the EVA/CORT microparticles, the release of CORT could not be directly determined from the TGA curves. Even more so, the finding of increased degradation temperature and increased weight loss percent suggests that the effects of processing on the microparticles and the effects of the *in vitro* experimental design prevented the interpretation of CORT release. Therefore, because of this unfortunate findings, the release of CORT from the microparticles could not be determined.

4.3 EVA/CORT Microparticle System

Considering the goal of this study, the conclusions regarding the preparation of the microparticles and the evaluation of CORT release do not support the successful preparation of an implantable microparticle system that could release CORT at a controlled rate. Until improvements are made, the EVA/CORT microparticle system would not operate as desired in order to control CORT release and elevation in rattlesnakes.

4.4 Recommendations

The conclusions listed above do shine light, however, on new improvements to the continuation of this study. Future research into the EVA/CORT microparticle system of this study would include two different components. First, the emulsification-solvent evaporation technique must include greater control of mixing conditions. A more precise

technique of adding the EVA+CORT stream to the emulsifier would produce more discrete microparticles that are of adequate size for use in rattlesnakes. Even more so, better control of dissolving the CORT into the polymer solution would thus increase the likelihood of the presence of CORT in the microparticles in the first place. This would then yield more intuitive results in characterization techniques to determine CORT presence.

The other component of recommendations for future research would include more direct characterization techniques. In order to determine possible interactions between the EVA and CORT, differential scanning calorimetry should be utilized in junction with FTIR. More importantly, direct methods of evaluating CORT release should be utilized. These methods would include techniques such as UV-vis spectroscopy, high-performance liquid chromatography, or perhaps enzyme-linked immunosorbent assay (ELISA) kits. These techniques could definitively determine whether or not CORT is being released, and if so, the efficacy of controlled release. These improvements would thereby enhance the viability of this microparticle design and moreover pave new exciting avenues for implants that can enable conservation biologists new tools for determining the effect of the stress response on animals.

Appendix A

Mixing Conditions

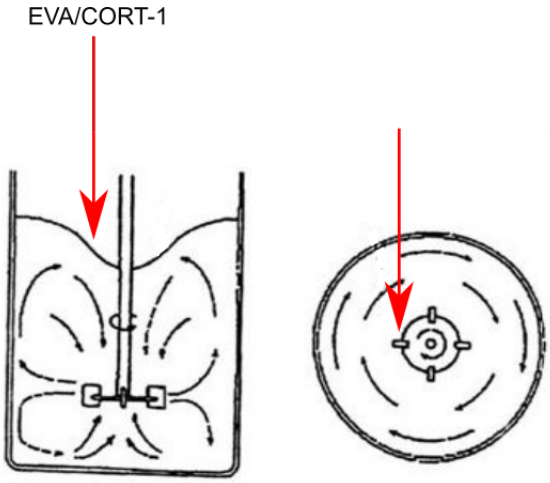


FIGURE A.1: Location of EVA+CORT stream added for EVA/CORT-1.

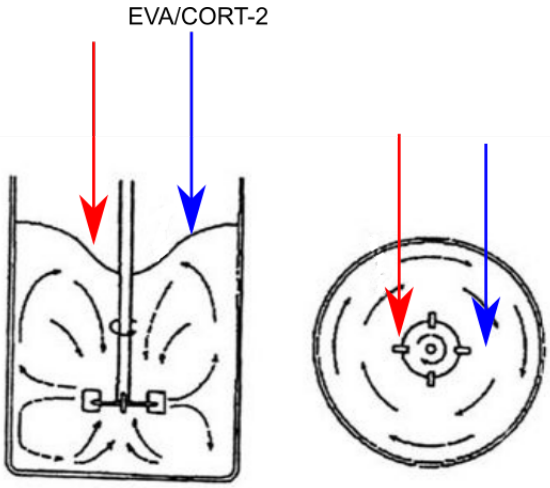
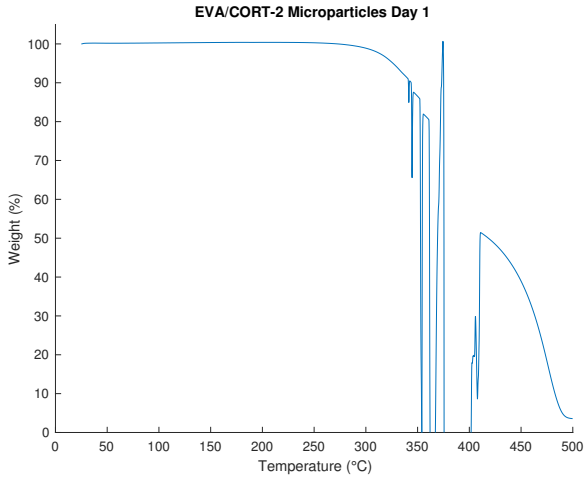
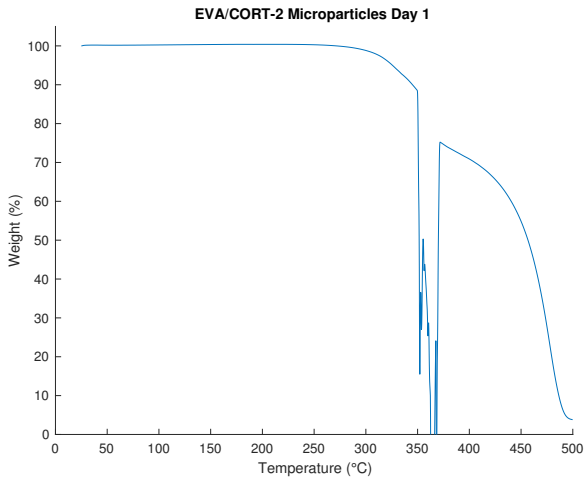


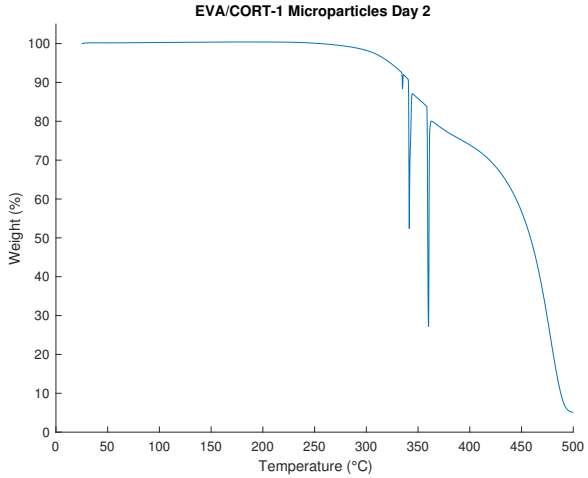
FIGURE A.2: Location of EVA+CORT stream added for EVA/CORT-2.

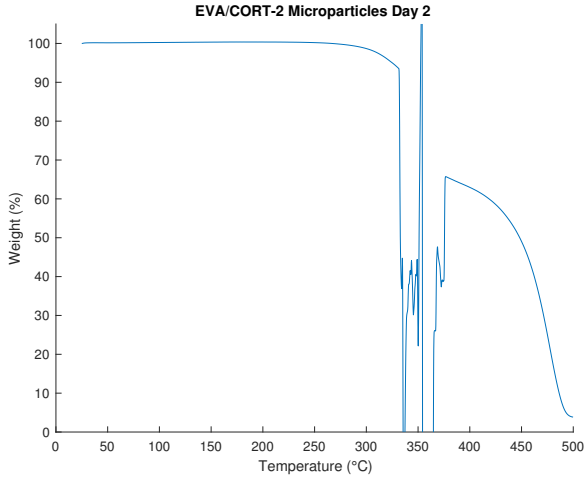
Appendix B

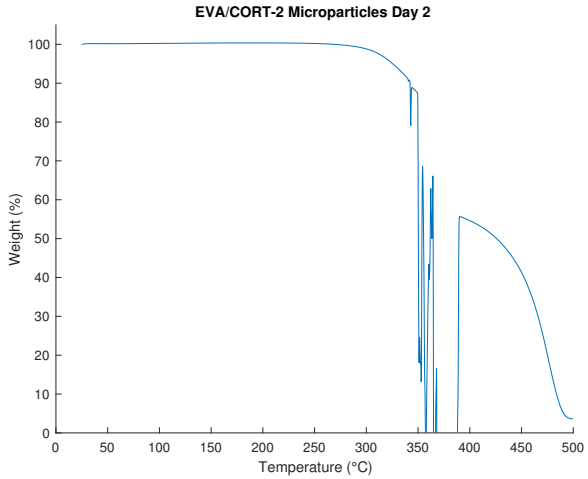
Aberration Plots

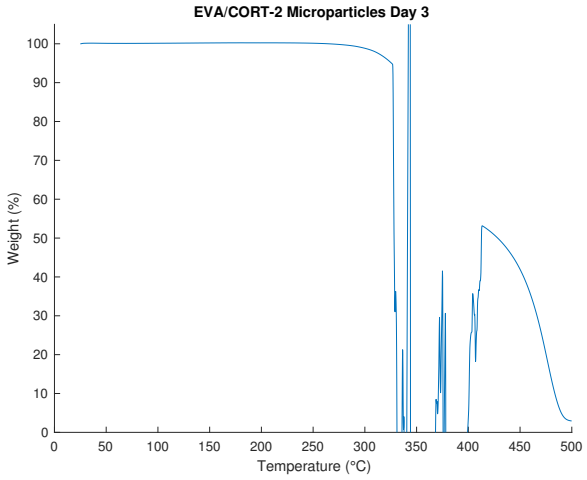


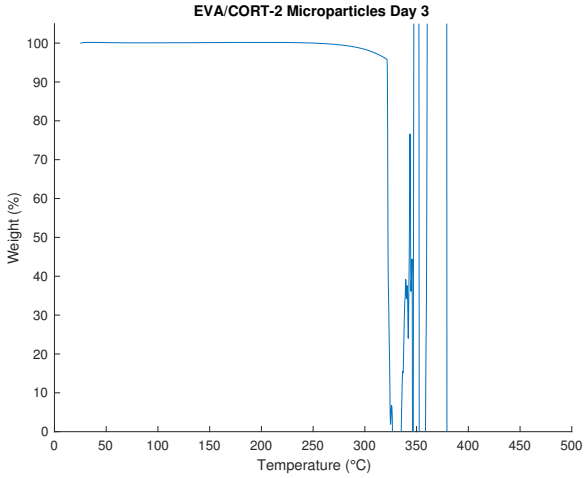



EVA/CORT-1 Microparticles Day 2
Weight (%)
Temperature (°C)
0
10
20
30
40
50
60
70
80
90
100
0
50
100
150
200
250
300
350
400
450
500


EVA/CORT-2 Microparticles Day 2
Weight (%)
Temperature (°C)
0
10
20
30
40
50
60
70
80
90
100
0
50
100
150
200
250
300
350
400
450
500


EVA/CORT-2 Microparticles Day 2
Weight (%)
Temperature (°C)
0
10
20
30
40
50
60
70
80
90
100
0
50
100
150
200
250
300
350
400
450
500


EVA/CORT-2 Microparticles Day 3
Weight (%)
Temperature (°C)
0
10
20
30
40
50
60
70
80
90
100
0
50
100
150
200
250
300
350
400
450
500


EVA/CORT-2 Microparticles Day 3
Weight (%)
Temperature (°C)
0
10
20
30
40
50
60
70
80
90
100
0
50
100
150
200
250
300
350
400
450
500

Appendix C

Bar Graphs

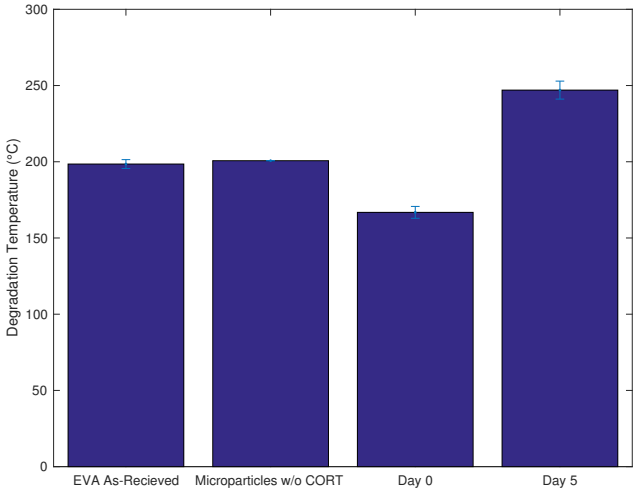


FIGURE C.1: Bar graph of degredation temperatures for different conditions of EVA/CORT-1 microparticles

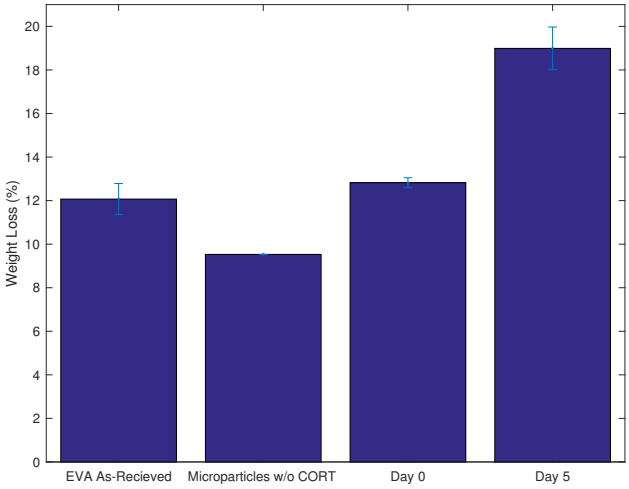


FIGURE C.2: Bar graph of weight loss percents for different conditions of EVA/CORT-1 microparticles

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