SILK ENCAPSULATED FILMS FOR SUSTAINED RELEASE OF BUPRENORPHINE

An honors thesis

Submitted by

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Abstract

A major problem with prescribed medication is patient compliance. In both the medical and veterinary field, physicians cannot be guaranteed that the patient will complete the prescribed regimen due to any number of factors. Buprenorphine (BUP) is a synthetic opioid with partial agonist and antagonist activity at the μ- and ORL- receptors and the κ- and δ- opioid receptors, respectively. BUP is used as an analgesic as well as a treatment for opiate addictions. Most analgesic or addiction treatment regimens tend to be inconvenient as they must be administered repeatedly for extended periods of time for any drug delivery route. With BUP, oral ingestion is not a desired route because the drug undergoes very high hepatic first-pass metabolism. For an oral administration of BUP, the desired dosage for the plasma must be increased tenfold, implying that 90% will become waste. For domesticated animals, there is no alternate route convenient for the pet owner. In humans on an outpatient basis, BUP is administered sublingually. For inpatient animals and humans, BUP can be administered intravenously, three times a day. In order to make BUP use more convenient and efficacious, a construct that will support zero-order, sustained release of BUP over an extended period of time must be developed.

An encapsulated film-reservoir was constructed by infusing BUP within a film made from silk-fibroin. The film was then coated with differing numbers of silk layers to control the release profile of the drug. These film-reservoirs were assayed *in vitro* via PBS assays. Because BUP is a Schedule III drug and large quantities may not be procured for *in vitro* use, Indigo Carmine (IC) was used as an analog and underwent the same *in vitro* assays to demonstrate comparability. The release profiles were then determined for various mass loadings (0.25 mg,

1.25 mg, 2.50) and various numbers of coatings (0x, 4x, 8x). Drug release from coated silk constructs was then modeled for zero-order release.

Indigo carmine did not demonstrate a significantly similar release profile to buprenorphine *in vitro*. Formulation of buprenorphine used and interactions between buprenorphine and silk require further investigations. Release profiles with increasing coating numbers demonstrated closer correlation to the kinetic model. The release profiles determined with these parameters were purely diffusion-based, however the addition of coatings approached zero-order release parameters when modeled. Mass loadings formed from indigo carmine in solution at a concentration of 5 mg/ml most closely followed the modeled profile. Films with 8x coatings demonstrated the slowest and most linear release kinetics.

Background and Introduction

The four most important goals in drug delivery systems are safety, stability, efficacy and convenience. In the veterinary field, it is difficult to develop medications that fulfill all of these goals, due to many factors including compliance and delivery pathway availability. For example, buprenorphine is a semi-synthetic opiate with various applications in the health field. In both the veterinary and medical field, it is used as an analgesic for patients who have undergone surgeries or who suffer from chronic pain. It is currently administered intravenously, intramuscularly, transdermally, and sublingually. In humans, it is not administered orally due to its high first-pass metabolism. For animals, however, in the case of prolonged administration, the only route convenient for pet owners is oral delivery. This is not the most efficient form of delivery, considering its high clearance by the liver. Additionally, the required dose for oral delivery of buprenorphine is ten times that of its injected form, which is not medically or cost effective considering that 90% of the administered drug would be ineffective. Furthermore, veterinarians cannot be guaranteed that the pet-owner will adhere to the regimen required for drug efficacy. Additionally, in humans, buprenorphine is used primarily as a treatment for opioid addiction, similarly to methadone, due to its partial agonist and antagonist activities; it has been proven to be more effective and less addictive than methadone in such treatments. In order for buprenorphine to work as a treatment for addiction, the patient must follow a long-term daily regimen, most often on an out-patient basis. As with pet-owner compliance, patient compliance may not be guaranteed.

While we currently have many means of managing pain in both humans and animals, the regimens required to attain a state of analgesia are not efficacious or convenient to the patient, animal, or pet-owner. Various drug delivery systems are in place that allow for different

pathways to be used, such as intravenous, subcutaneous, intramuscular, transdermal, gastrointestinal and sublingual routes. While all of these pathways have been proven to work, there are a number of imperfections for each. As previously stated, with regard to buprenorphine, the gastrointestinal pathway does not provide for efficient use of the dosage required, where a mere 10% of the administered drug passes through the liver's first-pass metabolism. Intravenous and intramuscular injections and sublingual routes must be repeated at least every eight hours for efficacy. Not only are these few available routes an inconvenience to the patient, animal, pet-owner, or physician but health professionals can neither be confident that the patient will return for injections or utilize the sublingual patches as directed, nor can they be confident that the pet-owner will care for the animal by following the necessary regimen. Therefore, it is necessary to develop a form of buprenorphine that will be more convenient to the patient, while also leaving the physician convinced that the patient or animal will receive the designated dosage.

I. Buprenorphine Uses, Pharmacokinetics and Pharmacodynamics

Buprenorphine (BUP), N-cyclopropylmethyl-7á-[1-(*S*)-hydroxy-1,2,2-trimethylpropyl]-6,14-*endo*-ethano-6,7,8,14-tetrahydronororipavine (Figure 1), is a highly potent and lipophilic, synthetic opioid with agonist and antagonist activity at the at the μ- and ORL1, and κ- and δ-receptors, respectively (Christoph *et al.,* 2005). It is a derivative of *thebaine*, which is a naturally occurring opioid (Craig & Stitzel, 1997). Drugs with agonist behavior bind to receptors on target cells to elicit a biological response from that cell, whereas drugs with antagonist behavior bind in order to prevent binding by other molecules (Katzung, 2004). Due to such activity at the opioid

receptors, BUP is utilized as a treatment for opiate addiction in a similar manner as methadone. Unlike methadone, however, buprenorphine shows only partial agonist activity at the μ- and ORL1 receptors (Robinson, 2002). This partial activity indicates that there is a "ceiling effect" on the agonist activity, meaning that as the dose increases, there will be a plateau of activity making overdose very difficult (Strain & Stitzer, 2006). Additionally, BUP dissociates slowly from the μ- receptor (Katzung, 2004). These behaviors reduce the abuse potential of buprenorphine when compared with methadone, which makes it a more attractive treatment course than methadone (Robinson, 2002). BUP has been shown to be less toxic and produce less physical dependence than other opioids used for addiction treatment (Robinson, 2002). Additionally, it has milder withdrawal symptoms as compared with methadone (Bose *et al.,* 2001). However, strong withdrawal symptoms do occur when taken concurrently with a strong acting opiate (Bennett & Brown, 2003). The high potency of buprenorphine allows for a much lower dosage when administered sublingually, where 8 mg of buprenorphine is comparable to 60 mg of methadone (Ahmadi, 2003). Furthermore, when used as an addiction treatment it has been demonstrated to reduce cravings for morphine and cocaine (Craig & Stitzel, 1997).

In addition to its use as a treatment for opiate addiction, buprenorphine is also a widely used analgesic, especially in veterinary areas (Martin *et al.,* 2001). It is a highly attractive analgesic because of its non-sedating, long acting behavior (Martin *et al.,* 2001). It is proven to be 30-50 times more potent than morphine and is efficacious for moderate to severe pain (Bose *et al.,* 2001). In a study of various pain models, Christoph *et al.,* (2005) found that BUP was more efficient than widely used painkillers such as morphine, ibuprofen, and gabapentin. Due to these advantages, buprenorphine is an especially popular choice for animal research. However, the current delivery systems of buprenorphine make its use rather cumbersome.

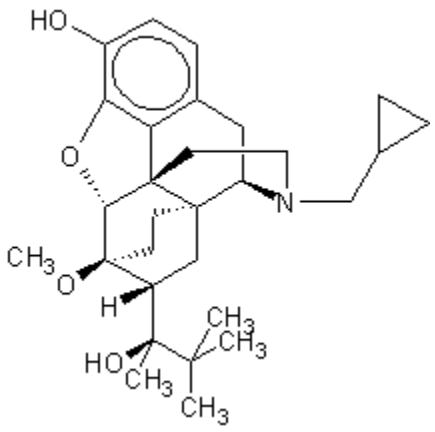


Figure 1 Structure of Buprenorphine

II. Buprenorphine Delivery Systems

While buprenorphine is an excellent analgesic and treatment for opiate addiction, its behavior with regard to potential for delivery systems must also be examined. The current modes of delivery that exist for buprenorphine are summarized in table 1. The most straightforward method available for delivery of buprenorphine is via intravenous injection, allowing for 100% bioavailability by definition (Robinson, 2002). Due to its bioavailability, intravenous injections are, comparatively, the most efficient form of buprenorphine delivery. However, analgesic effects last only 6-8 hours (Strain & Stitzer, 2006). Thus, should a patient need prolonged exposure to the painkiller, he or she would need to receive an injection at least every 8 hours. However, this can be avoided by the uses of sublingual tablets or liquids (Bennett & Brown, 2003), which can be held under the tongue for 3-10 minutes and 3-5 minutes, respectively, until they dissolve (Strain & Stitzer, 2006). Unfortunately, the sublingual route of administration lowers the bioavailablility of the drug by at least 40% (Strain & Stitzer, 2006). This indicates that a much higher dosage must be applied in order to achieve the same effect.

Due to buprenorphine's high lipophilicity, other routes of delivery are also being investigated, such as transdermal administration (Bose *et al.,* 2001).

Table 1 Summary of Current Modes of Delivery for Buprenorphine

Delivery Mode	Formulation	Advantages	Disadvantages
Intravenous injection (Reckitt Benckiser Pharmaceuticals Inc., 2007)	• 0.3 mg BUP HCl, 50 mg anhydrous dextrose, H_2O for injection	• 100% bioavailability • Rapid onset of action	• 3x daily administration • 4-8 hour duration of action • Inpatient use only • Invasive
Intramuscular injection (Reckitt Benckiser Pharmaceuticals Inc., 2007)	• 0.3 mg BUP HCl, 50 mg anhydrous dextrose, H_2O for injection	• 75% bioavailability • Rapid onset of action	• 3x daily administration • 4-8 hour duration of action • Inpatient use only • Invasive
Sublingual solution (Strain *et al.,* 2004)	• 2, 4, and 8 mg BUP, 40% ethanol	• 60% bioavailability • Outpatient use • Non-invasive • Rapid onset of action	• 3x daily • Increased abuse potential • Not applicable for veterinary field
Sublingual tablet (Strain & Stitzer, 2006)	• 4 and 8 mg BUP tablet	• Outpatient use • Non-invasive • Rapid onset of action	• 25% bioavailability
Transdermal – Experimental (Evans & Easthope, 2002)	• 20, 30, and 40 mg BUP in adhesive polymer matrix patch	• 72-hour release duration • Non-invasive • Maintains plasma concentration of BUP	• 50% bioavailability • Increased abuse potential • Not applicable for veterinary field • Causes skin irritation
Oral – Experimental (Martin *et al.,* 2001)	• 0.5mg/kg body weight BUP in gelatin	• Applicable for veterinary field	• 10% bioavailability

Another common mode of drug delivery is via oral consumption. Ordinarily, oral delivery is the most convenient form of administration, however, the interaction between

buprenorphine and the gastrointestinal system makes oral delivery rather ineffective (Robinson, 2002). Under the sublingual delivery mode, the liver metabolizes buprenorphine into the active metabolite norbuprenorphine via dealkylation by cytochrome P450 3A4 (Strain & Stitzer, 2006). However, when the drug passes through the gastrointestinal system, it undergoes significant hepatic first-pass metabolism (Bose *et al.,* 2001). First-pass metabolism is the clearing of drugs entering the liver through the portal vein by enzymes and/or secretion into bile (Katzung, 2004). Due to the first-pass effect, approximately 90% of buprenorphine is cleared by the liver before ever reaching the bloodstream (Martin *et al.,* 2001). Thus, the dosage for oral delivery must be increased tenfold from that of intravenous injection in order for buprenorphine to take effect. For example, the required dosage to achieve analgesia intravenously in male Long-Evan rats is 0.05 mg/kg of body weight, while that required for oral delivery is 0.5 mg/kg of body weight (Martin *et al.,* 2001). This makes for a rather inefficient use of the applied initial dosage. Unfortunately in the veterinary field, oral delivery is the only route available for long-term care as injections must be administered so frequently. Thus a controlled release form of buprenorphine is especially appealing to the veterinary community.

III. Controlled Release Drug Delivery Systems

In order to achieve sustained release of buprenorphine over a long period of time, a zero-order controlled release mechanism must be developed. A zero order mechanism signifies that the rate of drug released from the source will remain constant at the steady state (Pritchard *et al.,* 2010). Additionally, zero-order release mechanisms have rates of release independent of the amount of drug remaining in the body (Katzung, 2004). Currently, there are various options for

controlled release of pharmaceuticals including pump and reservoir implantations (Pritchard *et al.,* 2010). Pumps aptly achieve sustained drug delivery to the body but they have certain drawbacks. The current implantable pumps available are non-biodegradable, indicating that they will eventually require explantation or refills (Dash & Cudworth, 1998). Another option for implantable controlled release is a reservoir system. Reservoir systems use a compact form of the drug at its core, encapsulated in a permeable, non-degradable membrane (Dash & Cudworth, 1998). This membrane controls the release kinetics of the drug from the reservoir into the body (Dash & Cudworth, 1998). A common apprehension with reservoir systems is the possibility of "dose dumping," which occurs when a greater dosage than desired is released into the body, often resulting in the drug concentration rising above the toxic threshold (Dash & Cudworth, 1998).

Recently, developments have been made in the area of degradable reservoir systems. These are an especially attractive route for drug delivery because they eliminate the need for explantation of the reservoir (Dash & Cudworth, 1998). In these systems, rather than using a non-biodegradable encapsulation, inert polymers which are eventually cleared by the body are used as the coating (Dash & Cudworth, 1998). Various polymers currently used are elastin-like polymers, silk-elastin-like copolymers, coiled-coil and leucin-rich protein domains, β-sheet forming polymers, alanylglycine polymers, recombinant poly(glutamic acid) polymers, and silk-like polymers (Haider *et al.,* 2004).

IV. Silk for Drug Delivery

Silk fibroin, produced by the silkworm *Bombyx mori,* has become a prevalent polymer for controlled release studies due to its biocompatibility, biodegradability, and mechanical strength (Pritchard *et al.,* 2010). Recent studies comparing silk with other commonly used polymers indicated that silk is significantly more biocompatible as compared with PLA, PGA, and collagen as a biomaterial (Wang *et al.,* 2007). Silk is a protein containing repetitive sequences for both amorphous and crystalline domains (Hofmann *et al.,* 2006 & Haider *et al.,* 2004). These crystalline domains form β-sheets which aid in the highly controllable rate of degradation, making silk fibroin especially useful for controlled release (Wang *et al.,* 2007). These β-sheets provide high stability and mechanical strength which are unique to silk biomaterials (Wang *et al.,* 2007). Such control of biocompatibility, biodegradability, and mechanical strength make silk rather desirable because it enables control over the release profiles of drugs (Wang *et al.,* 2007). Pritchard *et al.,* (2009) found that encapsulating a packed powder tablet of adenosine in silk fibroin layers yielded desired results of linear release when assayed *in vitro*. We believe that by encapsulating buprenorphine in silk, similar release profiles will occur.

In addition to the finding that varying the number of coatings helps to control release rate, Hofmann *et al.,* found that the primary driving force for compounds released from silk films with zero coatings was the molecular weight of the loaded compound (2006). By using dextrans ranging from 4-44 kDa in molecular weight loaded into 5% silk films, Hofmann *et al.,* demonstrated that the larger the molecular weight, the slower the release rate from the silk film (2006).

V. Indigo Carmine as an Analog for Buprenorphine

Buprenorphine is classified as a Schedule III drug by the United States Drug Enforcement Administration. This classification is the result of three findings (21 U.S.C.):

A) "The drug or other substance has a potential for abuse less than the drugs or other substances in schedules I and II
B) The drug or other substance has a currently accepted medical use in treatment in the United States
C) Abuse of the drug or other substance may lead to moderate or low physical dependence or high psychological dependence"

Due to this classification, there are strict rules and regulations regarding the procurement and use of buprenorphine. Thus, it is necessary to find a suitable analog to buprenorphine that will demonstrate an *in vitro* release profile that is not statistically significantly different from that of buprenorphine. Based on the findings of Hofmann *et al.*, the compound should have a molecular weight that is similar to that of buprenorphine (2006). It should also have a similar solubility.

Indigo carmine, disodium (2E)-3-oxo-2-(3-oxo-5-sulfonato-1H-indol-2-ylidene)-1H-indole-5-sulfonate (Figure 2), is a dye that is commonly used for the testing of renal function, and the detection of chlorates and nitrates (National Center for Biotechnology Information [NCBI], 2007). Indigo carmine is similar to buprenorphine with regard to molecular weight, size and solubility. Buprenorphine has a molecular weight of 467.64014g/mol while indigo carmine has a molecular weight of 466.35286 g/mol, indicating a difference of only 0.275% from buprenorphine. Additionally, both compounds have solubilities of up to 10 mg/mL. Thus, indigo

carmine should serve as an adequate substitute for buprenorphine with regard to release from silk film constructs.

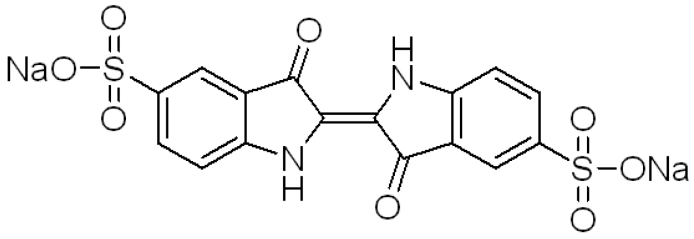


Figure 2 Structure of Indigo Carmine

VI. Kinetic Release Modeling

Ritger & Peppas (1986) demonstrated a mathematical model for drug release from polymeric films. The two basic types of diffusion often used for modeling purposes are Fickian diffusion, where release is based solely on the concentration gradient or Case-II diffusion, which demonstrates zero-order release (Ritger & Peppas, 1986). For these models, it is necessary that the release constructs are kept at the perfect sink condition, where the environment in which the drug is placed has a constant drug concentration of zero, indicating that the surfaces of the release device remains at a constant drug concentration (Ritger & Peppas, 1986). Fickian diffusion release from a thin polymer film can be represented by a mathematical equation given appropriate initial and boundary conditions :

$$\frac{M_t}{M_\infty} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2\pi^2} exp\left[\frac{-D(2n+1)^2\pi^2}{l^2} t\right] \quad (1)$$

Where M_t is the mass released at time t (mg), M_∞ is the mass released as $t \rightarrow \infty$ (mg), D is the diffusion coefficient (μm^2/day), assuming one-dimensional diffusion in the x direction and l^2 is

the thickness of the film (μm^2). Fickian diffusion can also be empirically represented as a function of the square root of time:

$$\frac{M_t}{M_\infty} = k'\sqrt{t} \tag{2}$$

where *k'* is the diffusion constant ($days^{-1}$).

Case-II diffusion, or zero-order diffusion, can be represented as a function of time:

$$\frac{M_t}{M_\infty} = k't \tag{3}$$

For polymeric slab modeling, researchers have found that most profiles fall between the cases for Fickian and Case-II diffusion, and are somewhat coupled systems (Ritger & Peppas, 1986).

$$\frac{M_t}{M_\infty} = k_1\sqrt{t} + k_2 t \tag{4}$$

This equation can be generalized as

$$\frac{M_t}{M_\infty} = kt^n \tag{5}$$

where *n* is the diffusional exponent and *k* is the diffusion constant accounting for the system ($days^{-1}$). The diffusional exponent can be employed to determine the type of release occurring in a system where Fickian diffusion occurs when *n=0.5* and Case-II diffusion occurs when *n=1* (Ritger & Peppas, 1986). Values of *n* occurring between these two values are considered mixed. While this empirical formula can serve as a good approximation for the transport mechanism, it is valid only for the first 60% of the fractional release of drug (Ritger & Peppas).

Hypothesis

In this study, we constructed silk films infused with buprenorphine or indigo carmine with varied numbers of silk coatings to allow for the sustained release of the compound over approximately one to two weeks. Thus, we hypothesized that by increasing the number of coatings applied to the constructs, the release profile can be altered to induce zero-order release of buprenorphine for a prolonged period of time as measured by absorbance of a PBS release assay. Furthermore, we hypothesize that indigo carmine will serve as an adequate analog to buprnorphine due to its similar solubility and molecular weight as measured by a PBS release assay and statistical analysis. Additionally, we believe that using these release profiles, we will be able to develop a sufficient release model for the constructs.

Specific Aims

Our three main objectives for this research experiment were:

1) *To demonstrate that indigo carmine is an adequate substitute for buprenorphine*. The objective of this aim is to demonstrate equivalency between buprenorphine and indigo carmine for the purpose of modeling buprenorphine drug release. To address this objective, 100 µL silk films will be loaded with 1mg of either indigo carmine or buprenorphine and coated with 2 or 4 coats of 8% silk. Since it has been demonstrated that the diffusion of compounds through silk is based on molecular weight, buprenorphine and indigo carmine should demonstrate statistically similar release profiles. This aim will be achieved when the release profiles for the two compounds fall within a statistically similar range.

2) *To develop the release profiles for silk constructs of varying indigo carmine concentrations and silk coating numbers.* The objective of this aim is to determine the release profile of indigo carmine from the silk constructs. This will be achieved by loading varying concentrations (1 mg/mL, 5 mg/mL, and 10 mg/mL) of indigo carmine into 0.250 µL, 8% silk films and applying different numbers of silk coatings (0, 4, and 8). Since silk has been previously shown to help control release profiles, it is expected that by increasing the number of coatings, the release profile will tend toward zero-order release.

3) *To adequately model the release of buprenorphine from the silk constructs.* The objective of this aim is to determine the release mechanism for the silk constructs. This will be achieved by applying the diffusion-based and empirical models set forth by Ritger & Peppas (1986) to each release profile attained with Specific Aim 2. It is expected that the release profiles will aptly fit to these models and tend toward Case-II diffusion with the addition of silk coatings.

Methods

Part I: Formation of Silk Constructs

A) Materials

Bombyx mori silk cocoons were obtained from Tajima Shoji Co., LTD (Sumiyoshicho, Naka-ku, Yokohama, Japan). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

B) Film Constructs

i) Silk Purification:

Cocoons of *Bombyx mori* were cut into dime-sized pieces and 5 g were boiled for twenty minutes in 2 L H_2O containing 4.24 g of sodium carbonate. The silk fibroin was then rinsed three times in pure water, each for twenty minutes. The fibroin was then pulled to separate and allowed to dry overnight at ambient conditions. The silk fibroin was then dissolved in a 9.3M LiBr solution at 60°C for 4 hours to yield a 20% (w/v) silk fibroin solution. The solution was then loaded into Slide-a-Lyzer 3-12 mL dialysis cassettes (MWCO 3,500, Pierce) and dialyzed against 1L distilled water per cassette in order to remove LiBr from the solution. The water was changed six times over a period of 2 days to maintain an adequate concentration gradient. The solution was removed from the cassettes, placed into 50 mL falcon tubes, and centrifuged twice in a fixed-angle rotator at 4-10°C and 8700rpm for twenty minutes to remove any debris. The concentration of the solution (w/v) was determined by evaporating the water from a known volume of silk and weighing the resulting product on a mass balance. The final silk solution was approximately 8% (w/v) and was stored at 4°C for future use.

ii) Silk Loading

Buprenorphine hydrochloride solution (1.0 mg/mL ± 5% in methanol) (MW = 504.10 g/mol) was obtained in 1 mL aliquots and dried at ambient conditions to remove the methanol. 8% silk fibroin solution was added to the dried compound to form a homogeneous solution with buprenorphine concentration of 10 mg/mL. Meanwhile, silk solution was added to the dye indigo carmine, which had been weighed by mass balance, to form homogeneous solutions with final indigo carmine concentrations of 1 mg/mL, 5 mg/mL, and 10 mg/mL. Buprenorphine solutions were stored at -20°C while indigo carmine solutions were stored at 4°C.

iii) Film Casting

Molds for silk film casting were constructed by applying a Teflon coating to the underside of the lids of both 48-well and 96-well tissue culture multi-well plates. 250 μL aliquots of each indigo carmine solution were distributed into the 48-well molds. 100 μL aliquots of the buprenorphine solution and 10 mg/mL indigo carmine solution were distributed into the 96-well molds. Control films were set for each film size with only silk solution. The films were allowed to dry overnight at ambient conditions. Once dry, the films were each submersed in 1 mL methanol for 5 minutes to promote greater β-sheet content. The methanol was then evaporated and the residue was resuspended 1 mL of Dulbecco's phosphate buffer saline (PBS) to quantify either buprenorphine or indigo carmine release during cross-linking, accordingly.

iv) Film Coating

Films were coated as previously described (Pritchard *et al.,* 2010). Briefly, each film was dipped into fresh 8% silk fibroin solution, set on the Teflon mold, and dried at 60°C for 30-45 minutes. The coating was then cross-linked in methanol for 5 minutes. This process was repeated

until the desired number of coatings had been reached. For all indigo carmine constructs, the methanol residues from the first coating were resuspended in 1 mL PBS and assayed for indigo carmine release to determine coating and cross-linking functionality. 100 µL films were coated 2x and 4x, while 250 µL films were coated 0x, 4x, and 8x. For all loading and coating pairs, n=3.

v) Characterization

Surface morphology and thickness of films and coatings were characterized by scanning electron microscropy (SEM). Films were snapped in half to expose cross-sections of the inner film and surrounding coatings. Image J imaging software was employed in order to determine the thickness of each coating. Samples were directly mounted and sputter coated with gold with a Ploaron SC502 Sputter Coater (Fison Instruments, UK). They were then examined at 15 kV.

Part II: Release Kinetics

A) Release Studies

i) Phosphate Buffer Saline Assay

Films were assayed in PBS as described previously (Pritchard *et al.,* 2010). Briefly, each film was immersed in 1 mL of Dulbecco's PBS and incubated at 37°C. Every twenty-four hours, the PBS was collected and immediately replaced with fresh PBS in order to mimic the perpetual sink conditions found *in vivo*. This process is diagramed in figure 3.

The PBS collected from each film with indigo carmine was placed in a clear-bottom 96-well plate, 100 µL per well, n=3 for each sample. The absorbance of indigo carmine concentration was measured at 610 nm and blanked with pure PBS using SOFTmax PRO 4.3 LS

software and a VERSAmax Tunable Microplate Reader (Molecular Devices). The PBS collected from each film with buprenorphine was placed in 1cm cuvettes, 1 mL per cuvette. The absorbance of buprenorphine concentration was measured at 286 nm using a UV spectrophotometer blanked with pure PBS.

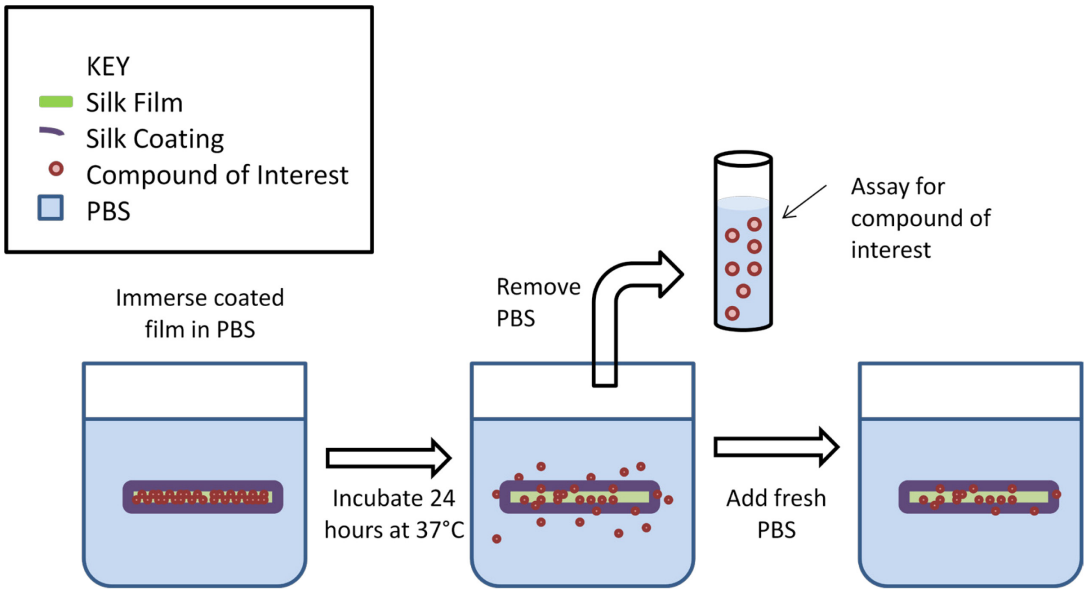


Figure 3 Diagram for PBS release assay. Adapted from Pritchard *et al.*, (2010)

ii) Remaining Mass Determination

The amount of indigo carmine remaining in each film was measured following release studies. Each exhausted film was immersed in 1 mL 9.3 M LiBr solution and incubated at 60°C for four hours in order to dissolve the silk and release any entrapped compound. 100 µL of each sample was placed into 900 µL of PBS for a 1:10 dilution. 100 µL of each solution was then transferred to a 96-well plate, n=3 for each specimen. The absorbance at 610 nm was then measured using the VERSAmax Tunable Microplate Reader blanked with 100 µL 9.3 LiBr in 900 µL PBS.

B) Release Modeling

i) Cumulative Release

Drug release was demonstrated as scatter plots between cumulative percent release as a function of time. Percent release was calculated as cumulative mass released over the mass released as $t \rightarrow \infty$ (M_t/M_∞). The total masses loaded were 0.25 mg, 1.25 mg, and 2.50 mg per film. Films were considered exhausted when the release rate decreased to less than 4 µg per day, which is below the therapeutic index for animal models employed for *in vivo* work.

ii) Release Modeling

Release of constructs was modeled first mathematically by equation (1). The values D and l^2 were grouped together to form the new value p (days^{-1}) where:

$$p = \frac{D}{l^2} \tag{6}$$

Equation (1) was encoded into MATLAB to produce models for release based on pure diffusion. Estimated p values were determined and were graphed as a function of the number of coatings.

The empirical model demonstrated in equation (5) was employed to show how closely related the determined release profile was to either Fickian or Case-II diffusion. The log of each side of equation (5) was taken in order to yield:

$$\log \frac{M_t}{M_\infty} = \log k + n \log t \tag{7}$$

The log of the release duration and the log of the fractional release were taken and plotted against each other. The linear trendline was calculated and the slope was found to be n.

Part III: Statistical Analysis

A paired t-test was performed to compare results from the films with buprenorphine to the 100 μL films with indigo carmine. A p-value of <0.05 was considered statistically significant and indicated that values for comparison were significantly different from each other.

For all cumulative release graphs, error bars represent the standard deviation. For graphs of p vs. coating number, error bars represent the confidence interval.

Results

SEM imaging of films coated 1x with 8% silk solution showed smooth surfaces and clear layers (Figure 4). Analysis of the layers showed a thickness of ~100 µm for the central film and ~90 µm per coating.

Preliminary studies measured the release of buprenorphine when embedded in 8% silk films and coated with 2 and 4 coatings (2x, 4x) of 8% silk. Absorbance was measured at 286 nm and a standard curve constructed using buprenorphine in PBS. Control films demonstrated absorbance at 286 nm indicating interference from degraded silk particles. Data from the control films and those containing buprenorphine were compared via a two-tailed paired *t*-test. p and t values for both 2x and 4x coatings were calculated (p=0.0014 and p=0.0005, respectively, df=11 for both). The interference from silk was accounted for by subtracting the average absorbance of control films from the measured absorbance of the films containing drug for each day of collection.

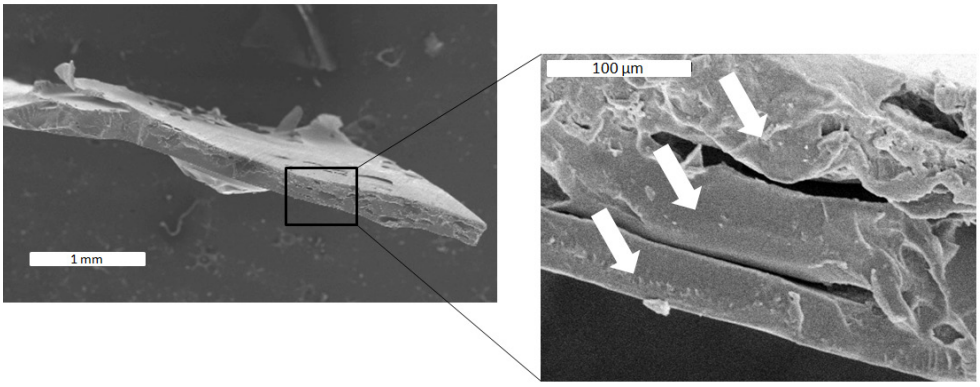


Figure 4 SEM image of silk film coated 1x with 8% silk

The release profile for buprenorphine films with 2x and 4x coatings in PBS fits a linear regression with high correlation (R^2=0.9879 and 0.9953, respectively) (Figure 5). Additionally, each release profile fits a logarithmic regression as well, however without as high a regression coefficient as with a linear regression (R^2=0.9445 and 0.9272). As demonstrated in Figure 4, the first day of collection demonstrates a large release of nearly 0.04 mg for both numbers of coatings. Following this initial burst, the release reduces to nearly 0.005 mg/day and 0.01 mg/day for 2x and 4x coatings, respectively, for the first five days. After five days, films coated twice released at a greater rate than those coated four times, ~0.08 mg/day and ~0.05 mg/day, respectively.

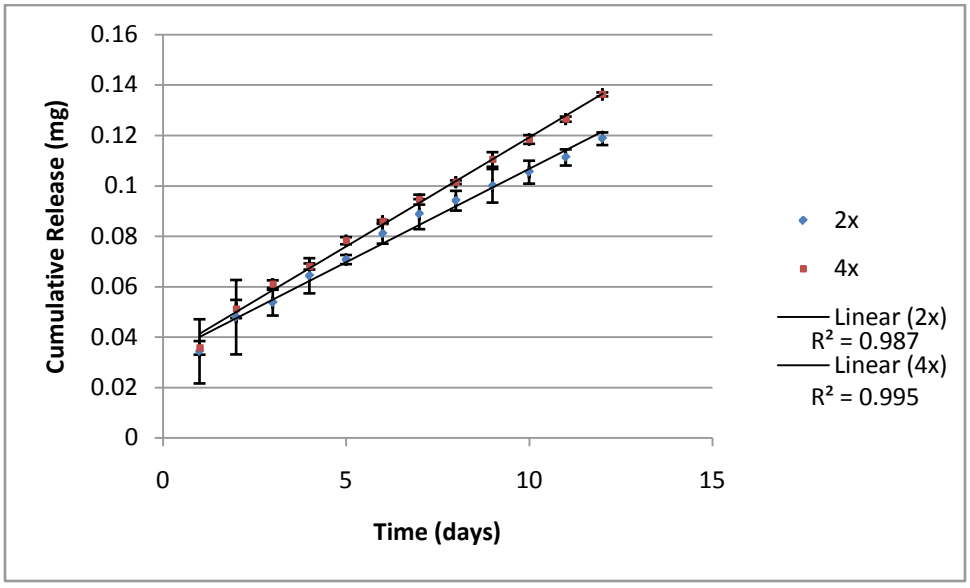


Figure 5 Cumulative mass release of buprenorphine in PBS. p=0.054, t=2.1530, df=11

Buprenorphine demonstrated slower and more linear release kinetics than indigo carmine, which is more logarithmic in shape. (Figure 6). An increase in coating from 2x to 4x produced more linear results for both indigo carmine and buprenorphine (Figures 5 and 6). When compared by paired, two-tailed t-test, films coated 2x had a p-value of 0.003 while films coated

with 4x had a p-value of 0.002. The burst effect for indigo carmine was lessened to an equivalent percentage of buprenorphine by the addition of two coatings to a total of four coatings.

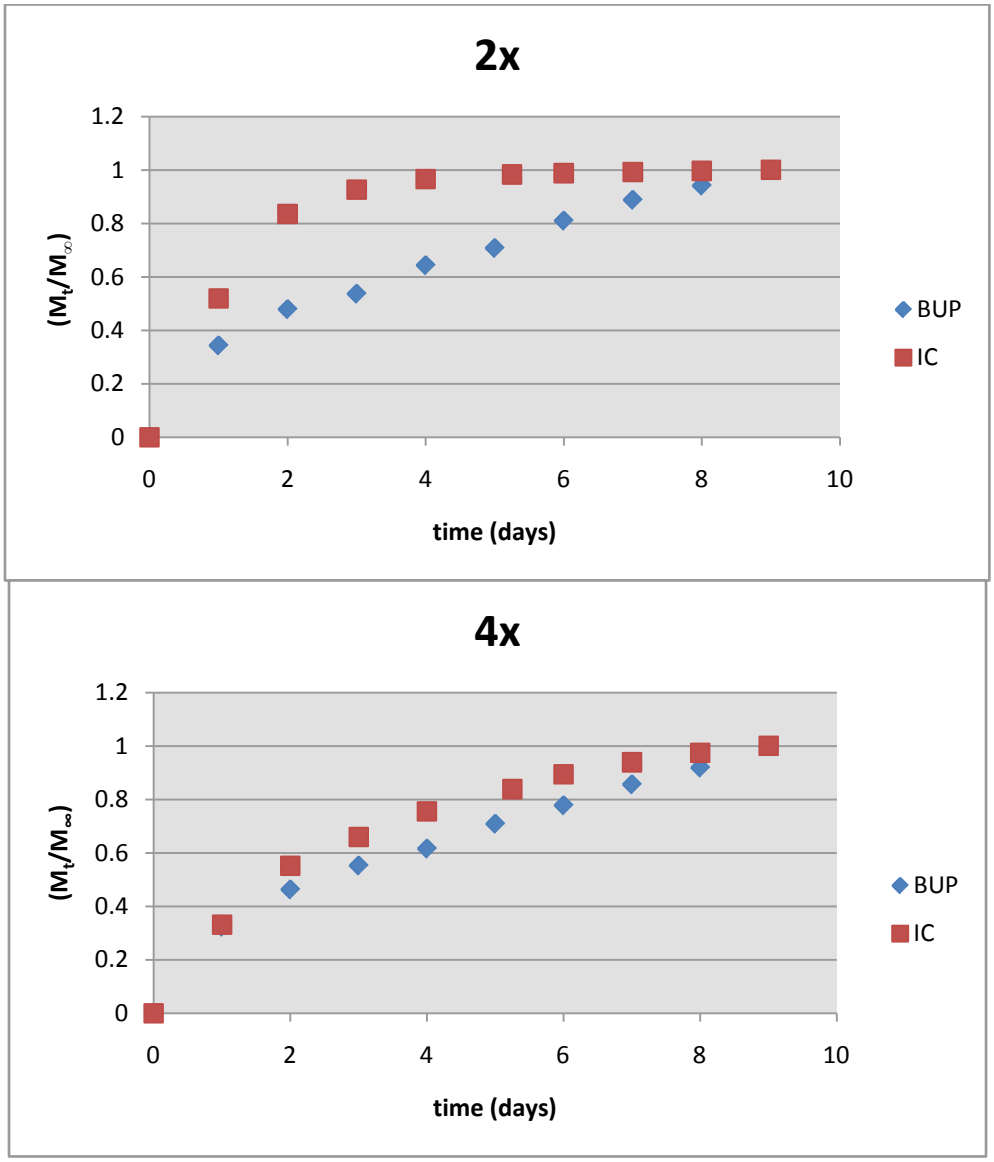


Figure 6 Comparison of buprenorphine and indigo carmine in terms of M_t/M_{inf}. 2x. p=0.003, df=8 4x. p=0.002, df=8

Mass of indigo carmine released from 8% silk films without silk coatings demonstrated dependence on the total mass loaded into the film. Figure 7 exhibits the cumulative release in

mass as a function of the time in days. Release without coatings fit more closely to a logarithmic regression rather than linear, with regression coefficients indicated in figure 7. With a regression value of 0.7859, the largest mass loading of 2.5 mg/film deviated most from the logarithmic trend. Increasing the initial mass loading led to an increase in the initial burst of indigo carmine from the films. Initial loadings of 0.25, 1.25, and 2.5 mg each had an average burst of 0.1, 0.3, and 1.5 mg, respectively. These bursts represented approximately 45, 25, and 60% of the initial mass loaded (Figure 7). Films loaded with 2.5 mg of indigo carmine showed the most variation in release for the first few days and then became more consistent in amount released.

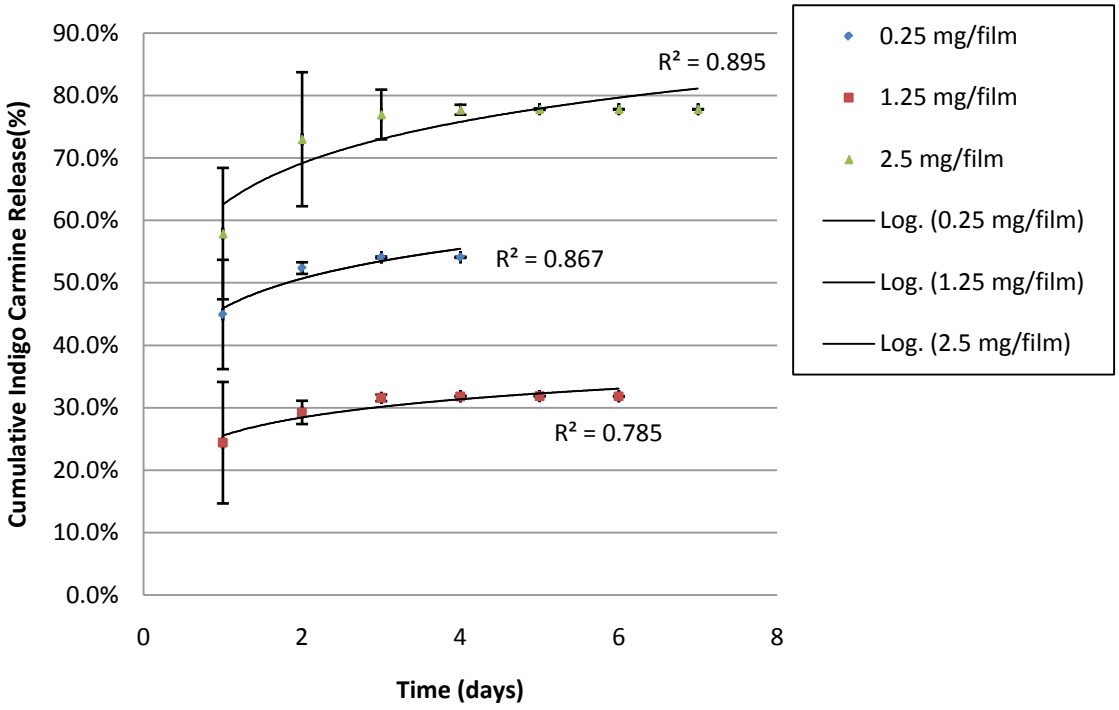


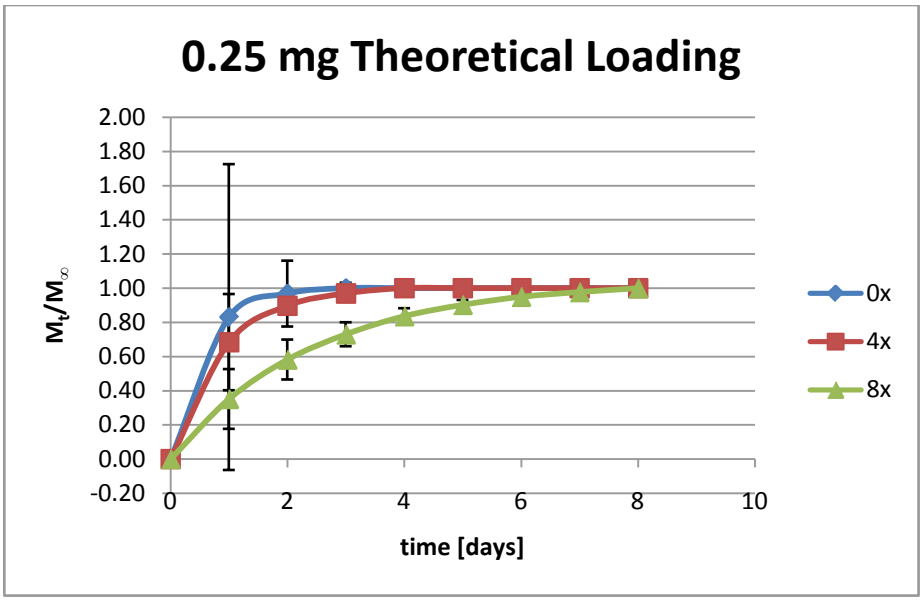
Figure 7 Cumulative indigo carmine released into PBS daily for 8% silk films with 0 coatings represented in percentage. Masses loaded are 0.25 mg/film, 1.25 mg/film, and 2.5 mg/film. Release percentage as compared with theoretical loading.

Films coated 0x ceased release of indigo carmine after differing days of collection, dependent on the initial mass loading. 0.25 mg loaded films exhausted on day 4, 1.25 on day 6,

and 2.5 on day 7. These films still apparently contained approximately 45%, 70% and 20%, respectively, of the initial loaded mass once release stopped.

The addition of coatings attenuated the logarithmic trend to a more linear-based model. Figure 6 demonstrates data collected for films theoretically loaded with 0.25 mg of indigo carmine. Films with 8 coatings released for the longest duration and had a longer t_{50}, or time until the cumulative release of 50% of the total release (Figure 7B). Films with higher numbers of coatings released more of the theoretically loaded dose of 0.25 mg and released at a slower rate than those with fewer coatings. Additionally, the initial burst was decreased by the addition of silk coatings to the construct.

A



B

Number of Layers	Point of Exhaustion (days)	R^2 (t=0 through point of exhaustion)	t_{50} (days)
0x	4	0.6347	0.3
4x	4	0.7557	0.4
8x	11	0.8384	1.8

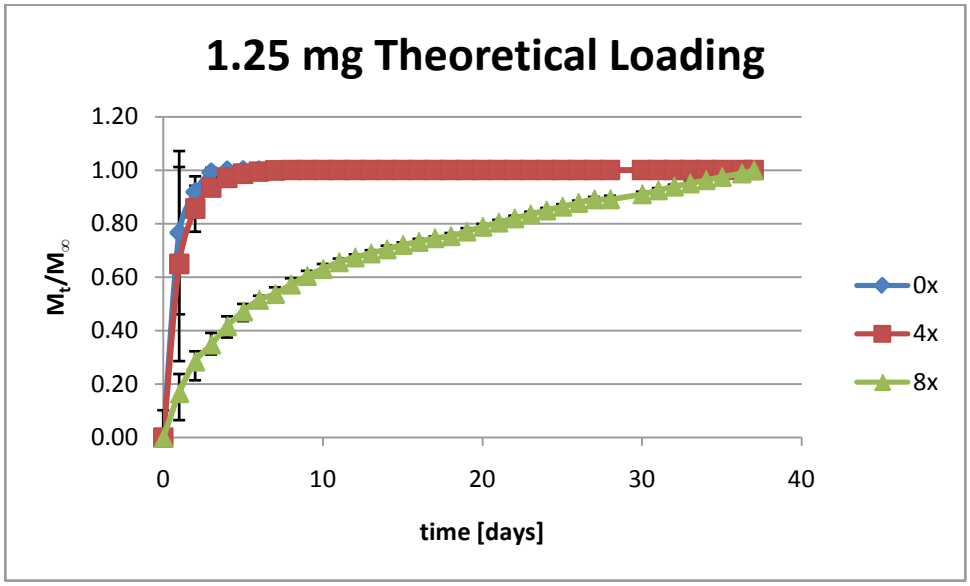
Figure 8 Cumulative release of indigo carmine in percentage of compound released for films theoretically loaded with 0.25 mg indigo carmine. A. Cumulative release as a percentage of the total mass loaded. B. Data based on A.

Increasing the initial mass loading led to an increase in t_{50} as well as a longer duration of release. Films theoretically loaded with 1.25 mg exhibited the longest duration of release, at 18 days (Figure 9). At the increased mass loading, regression coefficients decrease in linearity. Films loaded with 1.25 mg have less of a fractional portion of loaded compound being lost due to the initial burst.

Increasing the initial mass again to a total of 2.50 mg increased the release kinetics of the films (Figure 10). As with the other two mass loadings, release duration and t_{50} increased as the number of coatings increased. Point of exhaustion and t_{50}, however, decreased when compared

with a theoretical initial mass loading of 1.25 mg per film (Figures 9 and 10). Similarly, the increase in initial loading from 1.25 mg to 2.50 mg caused the trend to decrease in linearity with the regression coefficient decreasing from 0.8622 to 0.828, respectively. Interestingly, films coated 4x released indigo carmine for 4 days longer when loaded with 2.50 mg as compared with 1.25 mg.

A

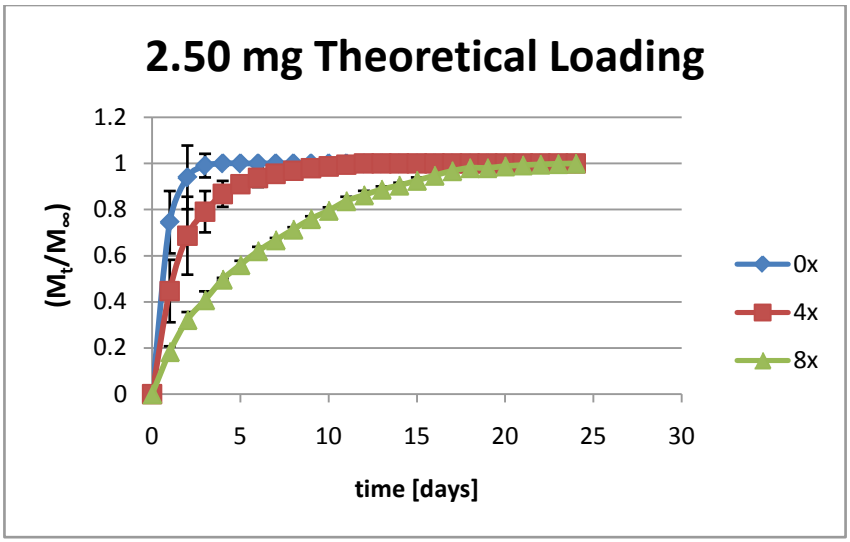


B

Number of Layers	Point of Exhaustion (days)	R^2(t=0 through point of exhaustion)	t_{50} (days)
0x	6	0.5545	0.5
4x	8	0.5598	0.8
8x	37	0.8622	6

Figure 9 Cumulative release of indigo carmine in percentage of compound released for films theoretically loaded with 1.25 mg indigo carmine. A. Cumulative release as a percentage of the total mass loaded. B. Data based on A.

A



B

Number of Layers	Point of Exhaustion (days)	R^2(t=0 through point of exhaustion	t_{50} (days)
0x	4	0.7028	0.3
4x	12	0.6297	1.2
8x	24	0.828	4

Figure 10 Cumulative release of indigo carmine in percentage of compound released for films theoretically loaded with 2.50 mg indigo carmine. A. Cumulative release as a percentage of the total mass loaded. B. Data based on A.

All films released some indigo carmine during film silk cross-linking (Figure 11). Films loaded with 1.25 mg indigo carmine released the most compound at approximately 0.058 mg, while films loaded with 2.50 mg released only 0.038 mg. Films loaded with 0.25 mg released the least at approximately 0.002 mg. During cross-linking of the first coat of 8% silk, indigo carmine release was reduced to effectively zero for all mass loadings.

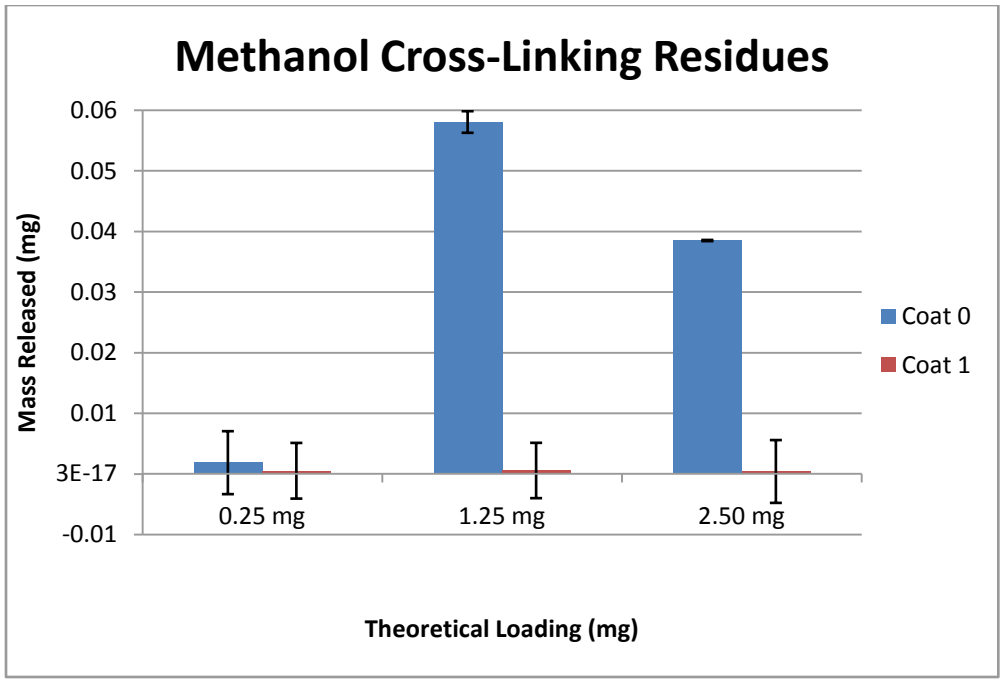
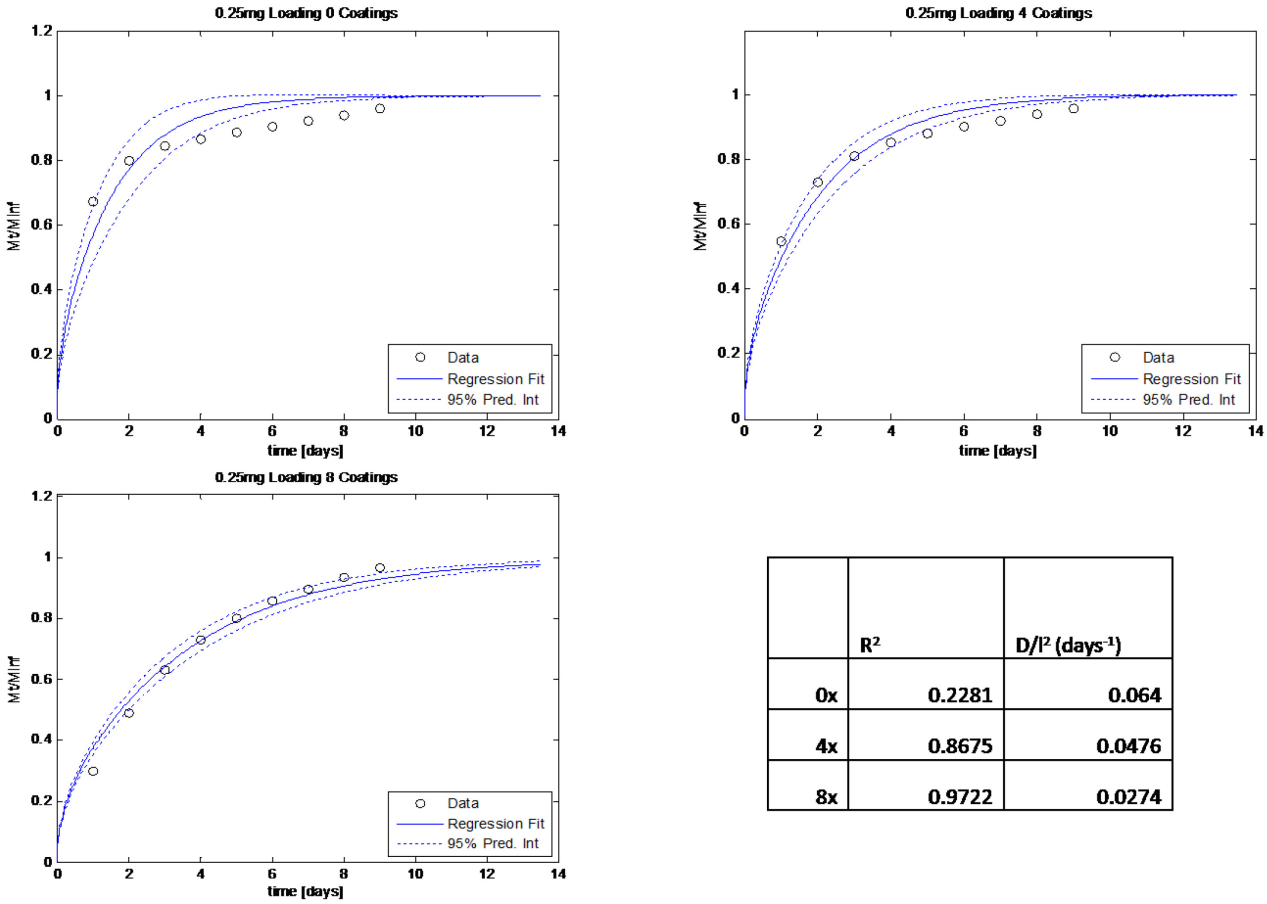


Figure 11 Mass of indigo carmine released during crosslinking of silk film and 1st silk coating.

Fickian diffusion situations were modeled based on equation (1) and are exhibited in Figures 12, 13 and 14. Films loaded with 0.25 mg indigo carmine and coated 0x with 8% silk show tremendously low correlation to the diffusion model, with a regression coefficient of 0.2281 (Figure 12). As the number of silk layers increases, the data fit more closely to the regression fit with 8x coatings having an R^2 value of 0.9722 (Figure 12).

Films loaded with 1.25 mg indigo carmine show better correlation to the diffusion model than those loaded with 0.25 mg indigo carmine. While those coated with 0 layers still showed the lowest regression coefficient of 0.8524, the increase in mass loading also caused a better fit to the model. Similarly to those loaded with 0.25 mg indigo carmine, an increase in coating numbers caused better correlation to the model.



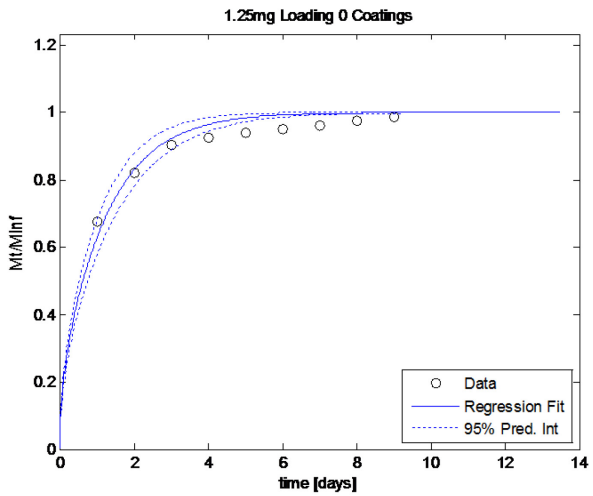
	R^2	D/l^2 ($days^{-1}$)
0x	0.2281	0.064
4x	0.8675	0.0476
8x	0.9722	0.0274

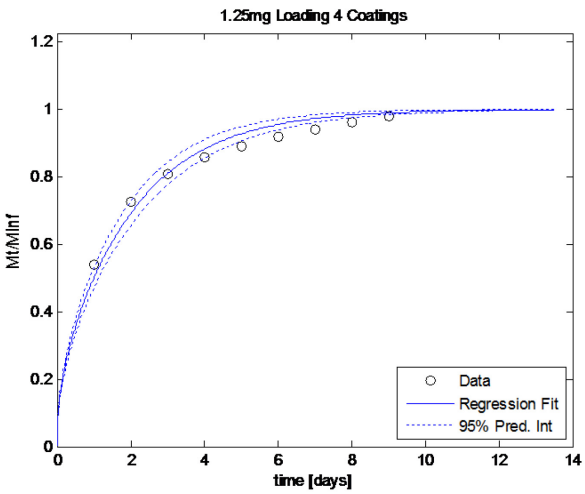
Figure 12 Diffusion based modeling for films loaded with 0.25 mg indigo carmine. Top left=0x, top right=4x, bottom left=8x, bottom right=modeling output.

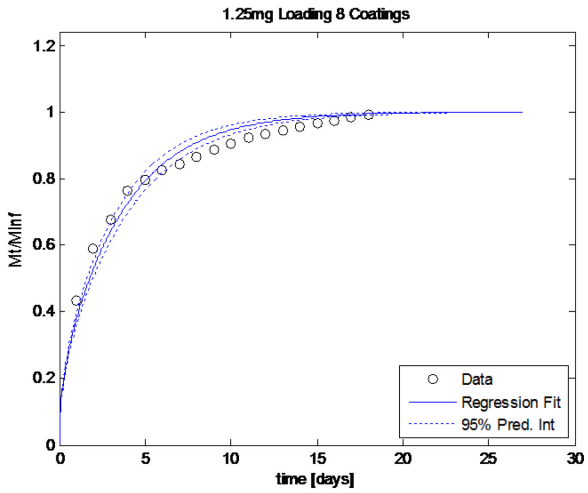
This trend does not remain true for films loaded with 2.50 mg indigo carmine, however (Figure 14). Contrary to films loaded with 0.25 mg and 1.25 mg indigo carmine, the films without coatings demonstrated the closest correlation to the diffusion model with a regression coefficient of 0.9786, as compared with that for 4x and 8x coatings of 0.818 and 0.8428, respectively.

Additionally, applying this diffusion model to the data attained provided estimates for the *p* value (D/l^2). As indicated in Figures 12-15, a general trend is formed in which the *p* value decreases as the number of coatings increases. The only anomaly to this trend exists for films

loaded with 2.50 mg, for which films coated 4x have a higher *p* value than those coated 2x with 8% silk – 0.0674 days^{-1} and 0.0645 days^{-1}, respectively.

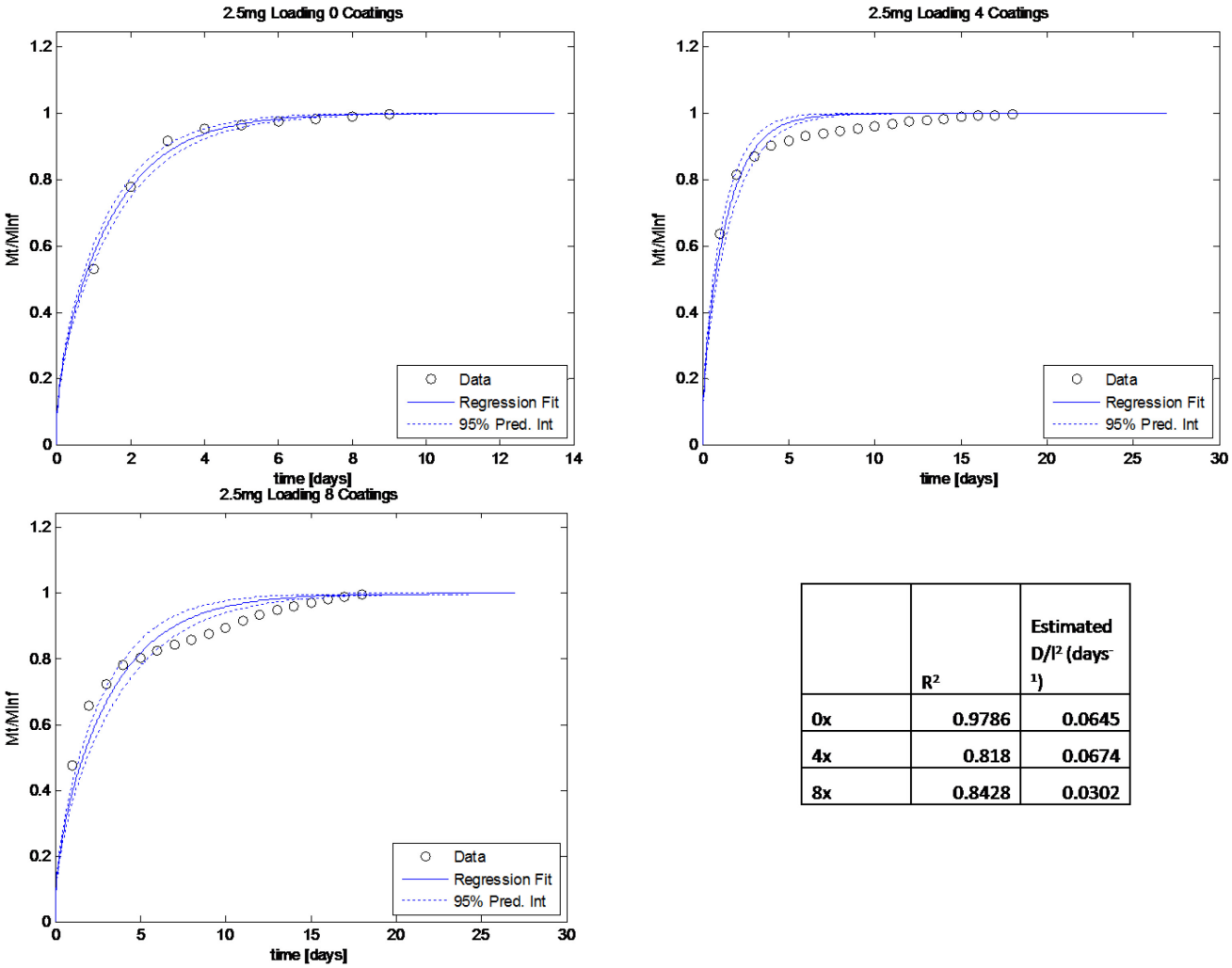






	R^2	Estimated D/l^2 (days^{-1})
0x	0.8524	0.0785
4x	0.9461	0.0486
8x	0.9408	0.0276

Figure 13 Diffusion based modeling for films loaded with 1.25 mg indigo carmine. Top left=0x, top right=4x, bottom left=8x, bottom right=modeling output.



	R^2	Estimated D/l^2 ($days^{-1}$)
0x	0.9786	0.0645
4x	0.818	0.0674
8x	0.8428	0.0302

Figure 14 Diffusion based modeling for films loaded with 2.50 mg indigo carmine. Top left=0x, top right=4x, bottom left=8x, bottom right=modeling output.

Figure 15 exhibits the trends between the diffusion coefficient in the form D/l^2 and the number of coatings applied to the films for each concentration. There is an overall trend of negative correlation between these two variables. For films loaded with 0.25 mg and 1.25 mg indigo carmine, the correlation fits a linear trendline with rather high regression coefficients of 0.9964 and 0.9899, respectively. Films loaded with 2.50 mg indigo carmine, however, could only be fit with a 2nd degree polynomial which has a maximum D/l^2 value at approximately 2 coatings before decreasing in value.

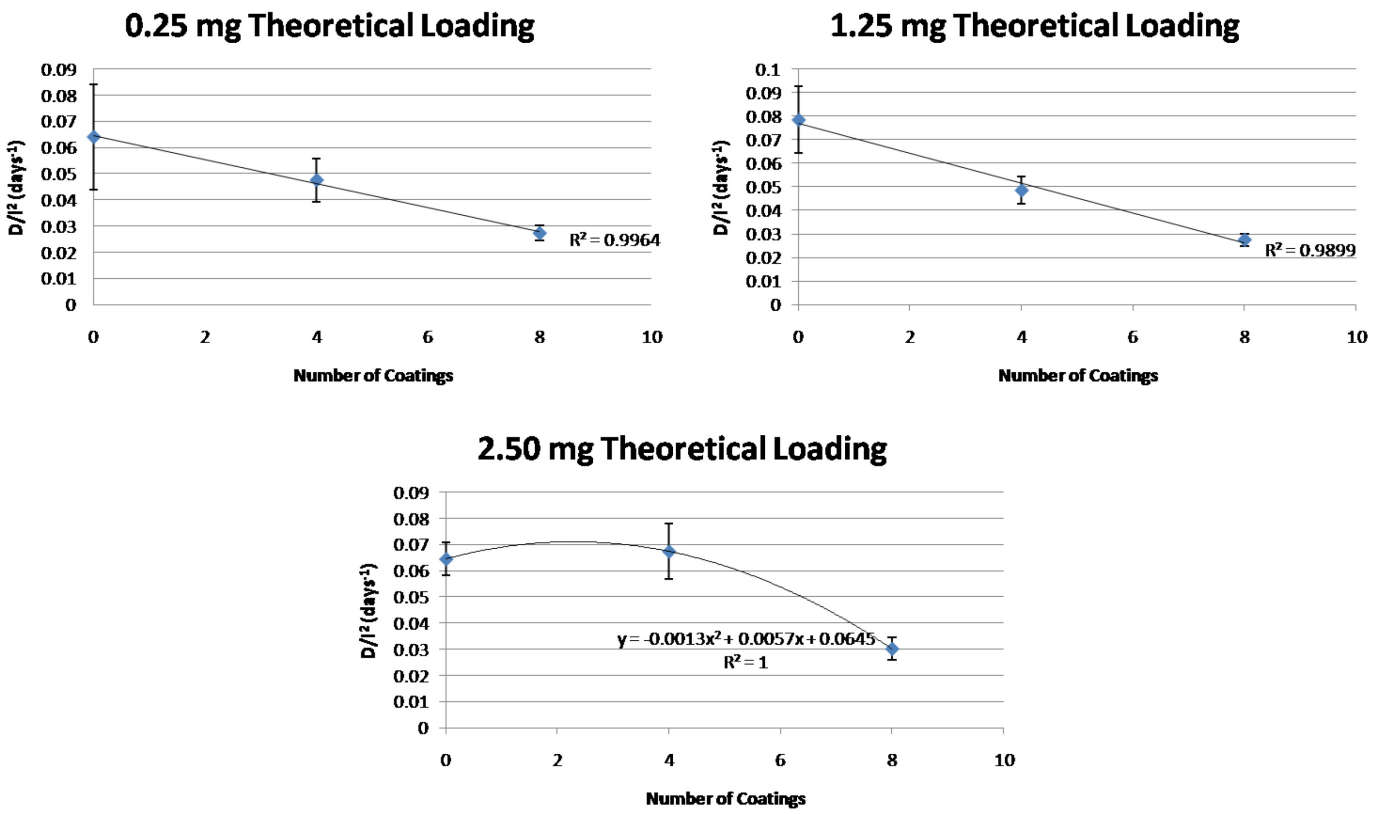


Figure 15 Trends for D/I^2 values. 0.25 mg and 1.25 mg theoretical loading fit with linear trendline. 2.50 mg theoretical loading fit with polynomial trendline (2nd degree).

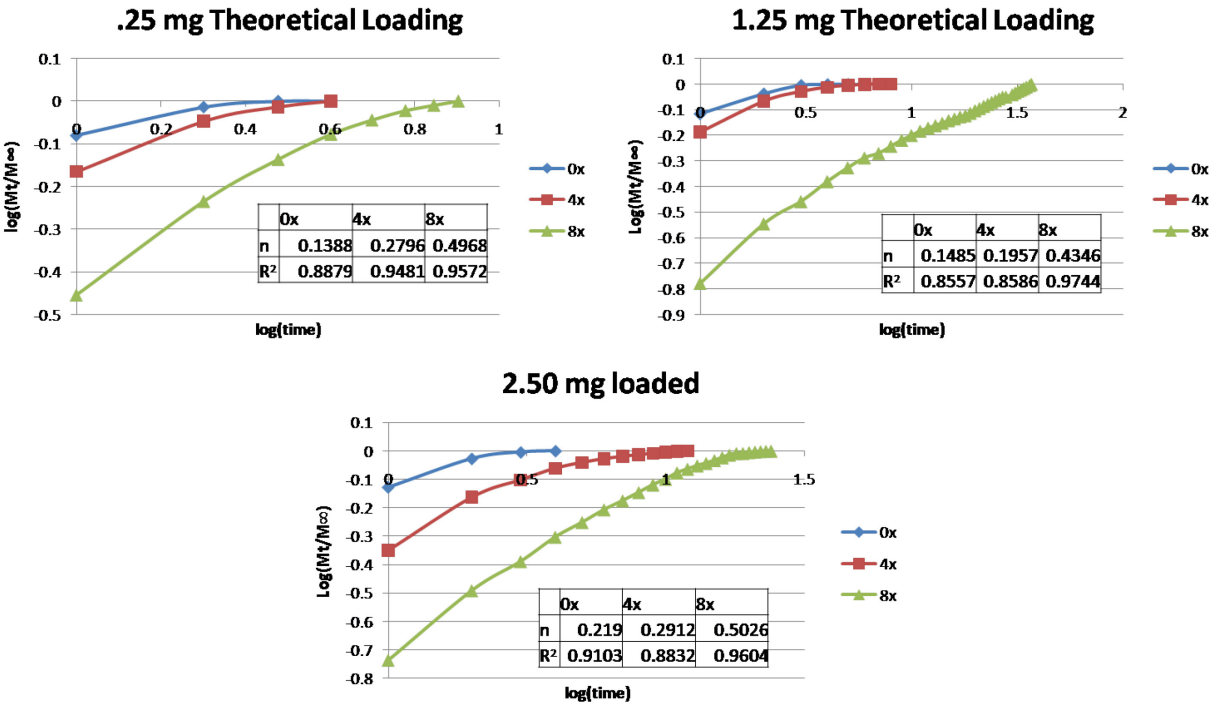


Figure 16 Empirical model for coupled Fickian and Case-II diffusion applied to various mass loadings and numbers of silk coatings.

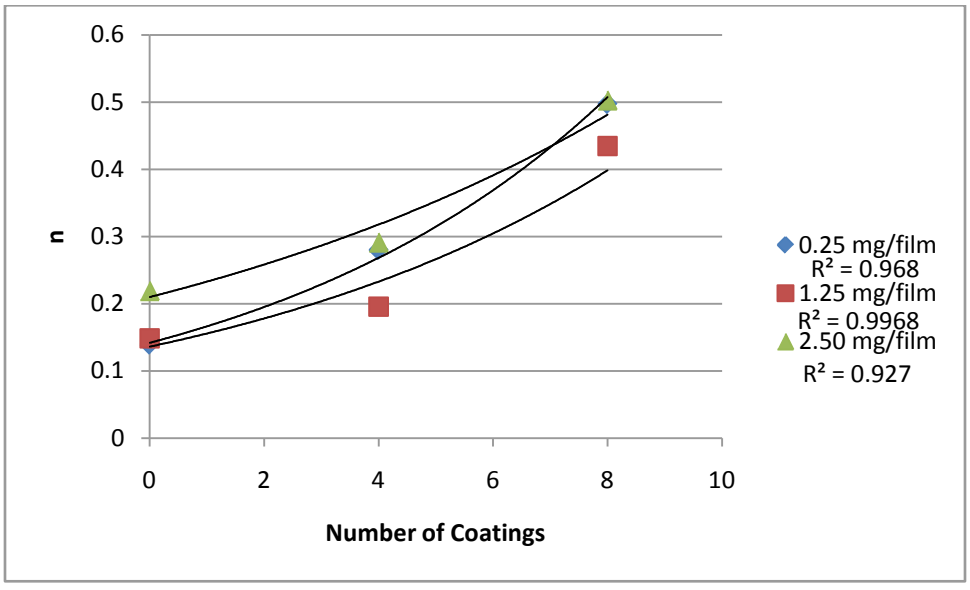


Figure 17 Diffusion coefficient, n, represented as a function of the number of coatings. Regression lines are based on exponential equations.

Application of the empirical model represented by equations (5) and (7) are shown in Figure 16. Regression coefficients represent correlation to a linear trendline. Generally, the regression coefficients increase with the numbers of coatings.

Furthermore, values for the diffusion coefficient, *n*, tend to increase with increasing numbers of coatings. Values for *n* converged toward 0.5 for films with 8x coatings, but did not rise above this value for the observed mass loading/coating pairings. As demonstrated in Figure 17, there is a positive correlation between n and the number of coatings for all mass loadings. Interestingly, these correlations are best represented by an exponential regression curve. Additionally, the value of *n* increases at a faster rate with the decrease in theoretical mass loading.

Discussion

Preliminary studies with buprenorphine demonstrated trends that disagree with those previously determined (Pritchard *et al.,* 2010). It is clear from figure 5 that silk films loaded with buprenorphine and coated 4x demonstrated a greater cumulative release and faster kinetics than those coated with 2x. Studies conducted previously exhibited a tendency for release kinetics and cumulative release from silk-coated constructs to decrease with the addition of layers (Pritchard *et al.,* 2010). While the paired, two-tail t-test p-value of 0.054 indicates that the differences between the release profiles of these two constructs are not significantly different, the trends do not support the previous findings that an increase in coating number alters the release profile and decreases the kinetics.

When compared with analogous films loaded with indigo carmine, release was shown to be on the same order of magnitude (Figure 6). The release profiles for both 2x and 4x coatings demonstrated a different release profile for the two compounds. These differences may be considered tremendously significant due to their p-values for 2x and 4x coatings of 0.002071 and 0.002925, respectively. Films loaded with indigo carmine demonstrated a release profile more closely related to a logarithmic regression, rather than that of buprenorphine, which was linear (Figure 6).

These discrepancies are cause for further investigation for the analogy between buprenorphine and indigo carmine. Because the formulation of buprenorphine utilized was actually buprenorphine HCl rather than pure buprenorphine, it is possible that the extra molecular weight added by the hydrochloric acid scaled down the release kinetics. It would be beneficial to determine whether this added weight is causing the variance in the release profiles.

Thus, it may be efficacious to add a filler of similar weight to the films with indigo carmine to determine if the hydrochloric acid is making the difference. It is also possible that the hydrochloric acid moiety or the buprenorphine itself is interacting with the silk film in some way that is causing slower release.

Since there is nothing in the literature describing any research employing buprenorphine and silk together, there is no way to conclusively state what is happening. It is highly probable however, that the silk protein is binding to the buprenorphine and hindering its release from the construct. Due to the high plasma protein binding of buprenorphine of 95-98%, we can hypothesize that buprenorphine is interacting with silk in a similar manner (Garret & Chandran 2006). This issue warrants more investigation to determine if there is an interaction between the silk and the buprenorphine.

As predicted, the addition of silk coatings to the constructs helped to slow the release rate for each of the mass loadings. While the release rates did not reach a perfectly linear trend in practice, compelling evidence suggests that with the addition of more coatings, zero-order release will be achieved (Figures 8-10). It is clear that with added coatings, the release profile alters from a logarithmic to linear shape. This finding is consistent with that of Pritchard *et al.,* (2010), who found that linear profiles were achieved through added coatings.

Interestingly, the amount of initial loading had a tremendous effect on the release profiles. The release profile was not independent of the mass loading, nor did it show a correlation to the added mass. To the contrary, the release profile was most linear when the film was set with indigo carmine in a 5 mg/mL solution with silk (1.25 mg loading). In fact, the profile for films set with a 10 mg/mL solution showed vast deviation from both predicted values

and those found with other concentrations. Because the maximum solubility of both buprenorphine and indigo carmine exists at 10 mg/mL, it is highly possible that the compound formed aggregates within the solution when films were set at this concentration. This hypothesis accounts both for burst effects during the release duration and for deviations from the expected profile.

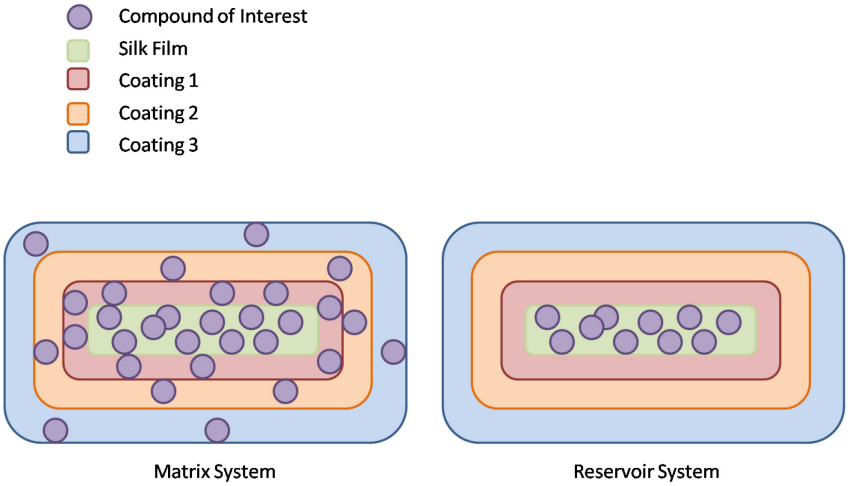


Figure 18 Diagram of release systems

For kinetic modeling purposes, it is interesting to determine whether the construct developed during these studies actually behaves more as a reservoir system or a matrix system. These two systems are diagrammed in figure 18. By studying the compound released during methanol cross-linking, we were able to determine that these constructs behave as a reservoir system. In a matrix system, the compound would diffuse into each new silk coating, forming a construct that has a gradient of compound concentration radiating from the center. In this type of system, compound would be released from the new coatings during methanol treatment. For these coated silk films, however, the indigo carmine residues from methanol treatment were

reduced to approximately zero during the first coating. Thus, little to no indigo carmine diffused into the new layers and this system was deemed a reservoir system.

With the type of system determined, kinetic modeling as described by Ritger & Peppas (1986) was performed. By applying the kinetic model for a polymer slab system (Equation (1)), we were able to deduce a relationship between the diffusion coefficient and the number of coatings. Results from the release studies fit this model very well with added coatings, as indicated by the regression coefficients, indicating that the system with these parameters is purely diffusion driven. As expected, as the thickness of the overall system increased by the addition of 8% silk coatings, the ratio D/l^2 decreased and the kinetics were scaled down, tending toward zero-order release (Figures 12-16). Furthermore, these relationships formed for initial loadings of 0.25 mg and 1.25 mg per film were almost perfectly linear when graphed against each other. This indicates that these relationships may be used, with high confidence, to determine the number of coatings to add in order to achieve zero-order release.

The empirical model utilized helped to indicate how close the systems were to zero-order release (equations 5 and 7). As expected, when the logs of the percent release and time were graphed against each other, films coated 8x produced the most linearity. Consistently with other results found in this study, the films loaded with 1.25 mg of indigo carmine and coated 8x correlated most closely with the linear regression fit. Interestingly, and consistent with the model for diffusion based release, the films with 8x coating appeared to be purely Fickian diffusion with n values of 0.4968, 0.4346, and 0.5026 for loading masses of 0.25 mg, 1.25 mg, and 2.50 mg, respectively (Figures 16 and 17).

A positive correlation was found to exist between the number of coatings and the diffusion exponent, *n* (Figure 17). This relationship most closely resembled an exponential trend, indicating that each additional coating helps to fast approach Case-II, or zero-order release.

Conclusions

At this point in time, we cannot conclusively state whether our first specific aim – proving that indigo carmine is a sufficient analog for buprenorphine – is supported. However, our preliminary studies with buprenorphine indicate that this specific drug has the potential for zero-order release from silk film constructs. The analogy between buprenorphine and indigo carmine must be further investigated in order to determine whether the drugs interact differently with silk.

We were able to achieve sustained release of indigo carmine from silk films coated 8x with 8% silk, however, the release profiles attained were logarithmic in nature rather than linear. The application of kinetic models proved that the addition of coatings to the silk construct pushed the release profile closer to a zero-order mechanism of release. Using these models, we determined that the optimal concentration for indigo carmine loading falls within the middle of the range of solubility for the compound, at 5 mg/mL. Furthermore, the kinetic models demonstrated that an increase in coating number causes both an increase in the diffusion exponent, n, which is indicative of approaching zero-order release, and a decrease in the diffusion constant, D, which indicates the deviation from Fickian diffusion.

Future Work

The first step for future work is to continue the investigation of indigo carmine as a sufficient analog for buprenorphine. Because it is rather difficult to attain buprenorphine for *in vitro* work, it is necessary to produce *in vitro* release profiles with a compound that behaves in a similar manner. This investigation should include the interaction between silk and buprenorphine, as well as release profiles for other formulations of the drug.

The next step for this study is to determine the optimal mass loading/coating number pairing for *in vivo* work. Following this, the constructs containing buprenorphine should be implanted *in vivo* to determine whether the zero-order release profile is both maintained and effective *in vivo*.

Rats are very popular models for analgesic quantification and qualification. Because variables are easy to control and specimen numbers are sufficient, rats are often chosen for *in vivo* studies (Cristoph *et al.,* 2005). A popular choice for rodent model is the Sprague-Dawley rat, which is an outbred rat that was originated in 1925 by the Sprague-Dawley Company in Wisconsin (Harlan Laboratories, Inc, 2009). The original colony was bred through a series of crosses between in single-hooded male and six albino females (Harlan Laboratories, Inc, 2009). These rats are albino and are often chosen for laboratory research due to their docile disposition, lending them to easy care and handling (Harlan Laboratories, Inc, 2009).

There are many well established pain assays for rodents as well, which makes determining the efficacy of analgesics very easy in these models (Cristoph *et al.,* 2005). The most commonly used assay for pain threshold is the tail flick test. There are two types of tail flick tests – hot water and heat lamp. The principle for each is the same in which a pain source is supplied to the tail, and the time it takes for the specimen to remove its tail from the source is

recorded (Cristoph *et al.,* 2005 & Martin *et al.,* 2001). Additional assays for analgesia in rats include various other pain models such as the formalin test for chemically induced persistent pain, the mustard-oil assay for visceral pain, the streptozotocin model for neuropathic pain, and the Randall Selitto test for inflammatory pain (Cristoph *et al.,* 2005). Thus, rats are highly useful models for nociception due to their ease of handling and the various assays for analgesia to which they may be subjected.

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