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Development of a Multilayered Association Polymer System for Sequential Drug Delivery

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DEVELOPMENT OF A MULTILAYERED ASSOCIATION POLYMER SYSTEM FOR SEQUENTIAL DRUG DELIVERY

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Engineering
at the University of Kentucky

By
Sharath kumar Chinnakavanam Sundararaj

Lexington, Kentucky

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Lexington, Kentucky

2013

ABSTRACT OF DISSERTATION

DEVELOPMENT OF A MULTILAYERED ASSOCIATION POLYMER SYSTEM FOR SEQUENTIAL DRUG DELIVERY

As all the physiological processes in our body are controlled by multiple biomolecules, comprehensive treatment of certain disease conditions may be more effectively achieved by administration of more than one type of drug. Thus, the primary objective of this research was to develop a multilayered, polymer-based system for sequential delivery of multiple drugs. This particular device was designed aimed at the treatment of periodontitis, a highly prevalent oral inflammatory disease that affects 90% of the world population. This condition is caused by bacterial biofilm on the teeth, resulting in a chronic inflammatory response that leads to loss of alveolar bone and, ultimately, the tooth. Current treatment methods for periodontitis address specific parts of the disease, with no individual treatment serving as a complete therapy.

The polymers used for the fabrication of this multilayered device consists of cellulose acetate phthalate (CAP) complexed with Pluronic F-127 (P). After evaluating morphology of the resulting CAPP system, *in vitro* release of small molecule drugs and a model protein was studied from both single and multilayered devices. Drug release from single-layered CAPP films followed zero-order kinetics related to surface erosion property of the association polymer. Release studies from multilayered CAPP devices showed the possibility of achieving intermittent release of one type of drug as well as sequential release of more than one type of drug. Mathematical modeling accurately predicted the release profiles for both single layer and multilayered devices. After the initial characterization of the CAPP system, the device was specifically modified to achieve sequential release of drugs aimed at the treatment of periodontitis. The four types of drugs used were metronidazole, ketoprofen, doxycycline, and simvastatin to eliminate infection, inhibit inflammation, prevent tissue destruction, and aid bone regeneration, respectively. To obtain different erosion times and achieve appropriate release profiles specific to the disease condition, the device was modified by increasing the number of layers or by inclusion of a slower eroding polymer layer. In all the cases, the device was able to release the four different drugs in the designed temporal sequence. Analysis of antibiotic and anti-inflammatory bioactivity showed that drugs released from the devices retained 100% bioactivity.

Following extensive studies on the *in vitro* sequential drug release from these devices, the *in vivo* drug release profiles were investigated. The CAPP devices with different release rates and dosage formulations were implanted in a rat calvarial onlay model, and the *in vivo* drug release and erosion was compared with *in vitro* results. *In vivo* studies showed sequential release of drugs comparable to those measured *in vitro*, with some difference in drug release rates observed. The present CAPP association polymer-based multilayer devices can be used for localized, sequential delivery of multiple drugs for the possible treatment of complex disease conditions, and perhaps for tissue engineering applications, that require delivery of more than one type of biomolecule.

KEYWORDS: Multiple drug delivery, Periodontitis, Cellulose acetate phthalate, Pluronic F-127, Sequential drug release, *in vitro* drug release, *in vivo* drug release.

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Table of Contents

List of Tables

List of Figures

Chapter 1 Introduction

The human physiology is an intricate system involving a network of many organs and organ systems. Human body works and maintains its homeostatic condition depending on complex cascade of events with several signaling molecules, involving more than one organ or a system. In the event of an injury or disease the physiological response required to bring the body back to normal condition also involves several factors working in an interrelated manner. One such condition which requires more than one factor that needs to be addressed for treatment is periodontitis. Treatment of this condition involves eradicating the microbial infection, controlling the inflammatory response, preventing bone resorption and aiding bone regeneration. The main aim of this research was to develop surface erosion based implantable polymeric device capable of delivering more than one type of drug in the required temporal sequence for treatment of such complex medical condition. Chapter 2 examines the background and significance of the releasing multiple drugs in the sequential order and also explores its usefulness in treatment of periodontitis. The details of the previous research and the use of surface eroding polymer used in this research are discussed. The types of drugs that are delivered using this device and their relevance in regard with the treatment of periodontitis are also briefly explained.

General mass loss and drug release properties of the association polymer system (CAPP) comprising of cellulose acetate phthalate (CAP) and Pluronic F-127 is analysed in chapter 3. Different small molecule drugs and a model protein were loaded in the CAPP films at different doses and their zero order release was studied. The fabrication of CAPP films in the form of multilayered devices for intermittent and sequential release of drugs are also discussed in chapter 3. These drug release profiles are predicted using a mathematical model and compared with the actual release profiles. Chapter 4 involves the fabrication of the CAPP multilayered device capable of releasing four drugs released in a sequential order, specifically aimed at the treatment of periodontitis. This included release of antibiotics, anti-inflammatory, anti-resorptive and osteogenic drug in the required temporal sequence. The bioactivity analysis of the released drug were performed and the multilayered device was also modified to obtain different erosion and release times by inclusion of slower eroding polymer layer.

Chapter 5 presents the *in vitro* and *in vivo* sequential release of four different drugs from the multilayer CAPP device. The *in vivo* drug release studies were performed by implantation of the multilayer device in the rat calvarium model. Multilayer CAPP device capable of releasing four different drugs in a sequential manner *in vivo* in different doses and erosion times were studied and compared with *in vitro* release. Based on the comparison of *in vitro* and *in vivo* release profiles, the possible changes that might be necessary in the device to achieve the appropriate *in vivo* release profile for treatment of specific disease conditions are also discussed.

The ability of this CAPP based multilayer device for delivering more than one type of drug sequentially is further discussed in the conclusion, along with its possibility to serve as a complete treatment for periodontitis. This device will not only be useful in the treatment of periodontal disease, it will also serve as a model for fabrication of devices with the general capability for delivering multiple drugs for treatment of complex disease conditions and for tissue engineering.

Chapter 2 Background and Significance

2.1 Treatments requiring multiple drug delivery

Pathogenesis of a disease condition is based on cascade of events with more than one type of biomolecules taking part in them. This creates the need for administration of multiple drugs for better and complete treatment of a particular disease condition. Some of the examples include severe bacterial infection required combination of antibiotics (Dowling 1957), infection accompanied by tissue loss (Younger, Duncan et al. 1998), cancer therapy (Wang, Rosano et al. 2010) and periodontitis (Rosen 2001). In the case of bacterial infection and biofilm formation more than one type of antibiotic agent may be required for complete elimination of infection (Griffiths, Ayob et al. 2011). Administration of more than one type of antibiotic would help fight different types of bacteria present and might also avoid the chances of bacteria developing resistance towards a particular drug (Dowling 1957, Griffiths, Ayob et al. 2011). Similarly in the case of bacterial infection which is accompanied with tissue loss, we need antibiotics to treat the infection followed by growth factors to aid tissue regeneration (Younger, Duncan et al. 1998). Another similar type of condition is periodontitis, an inflammatory condition caused due to bacterial infection that ultimately leads to tissue loss (Rosen 2001, Polimeni, Xiropaidis et al. 2006), would require antibiotics, anti-inflammatory and osteogenic agents for complete treatment. There is a growing body of research which suggests the need for multiple drug delivery or combinatorial drug therapy for effective cancer treatment (Chen and Jin 2010, Wang, Rosano et al. 2010, Cao and Bae 2012, Lee and Nan 2012). The process of wound healing also shows the presence multiple stages such as hemostasis, inflammation, proliferation and wound remodeling involving multiple biomolecules (Gurtner, Werner et al. 2008, Velnar, Bailey et al. 2009).

Along with the treatment of the above mentioned conditions, more and more research indicate the need for multiple growth factors for tissue engineering applications. The importance of multiple growth factors for effective tissue regeneration has been reviewed by Chen et al. (Chen and Jin 2010). There are more of these studies on tissues like bone, cartilage and blood vessels. Studies have shown the effect of mixture of growth factors on increase in number of human alveolar bone cells *in vitro* (de Oliveira, de Oliva

et al. 2008). Yeh et al., showed the synergistic effect of osteogenic protein 1 and interleukin-6 (IL-6) in stimulating differentiation of rat osteoblastic cells (Yeh, Zavala et al. 2002), and the combined effect of insulin like growth factor I (IGF-I) and transforming growth factor (TGF-β) on chrondrogenesis *in vitro* was shown by Fukomuto et al. (Fukumoto, Sperling et al. 2003). The well-studied process of angiogenesis also shows the presence of multiple growth factors working together in a complex cascade of events starting with vasculogenesis (vessel formation), followed by angiogenic remodeling and maturation of blood vessels (Yancopoulos, Davis et al. 2000). Even for regeneration of nervous tissue and guidance of axons there are multiple factors involved (Dontchev and Letourneau 2003). For regeneration of more complex tissues like muscles both angiogenesis and myogenesis growth factors would be required (Borselli, Storrie et al. 2010).

The above examples show the need of multiple drugs for complete treatment of a condition or multiple growth factors for regeneration of a tissue. Growth factors are expressed in a time-dependent manner during the process of tissue regeneration. The sequential expression of growth factors in differentiation of osteoprogenitors to osteoblasts *in vitro* was investigated by Huang et al. (Huang, Nelson et al. 2007). The process of bone fracture healing (Cho, Gerstenfeld et al. 2002) and tendon to bone healing (Würgler-Hauri, Dourte et al. 2007) also involves temporal expression of several growth factors. Sequential delivery of growth factors has also shown to have positive effect on cartilage tissue engineering (Martin, Suetterlin et al. 2001, Worster, Brower-Toland et al. 2001, Pei, Seidel et al. 2002). Sequential expression of growth factor can also be observed in the case of wound healing as seen in the spatiotemporal expression of periostin during skin development (Zhou, Wang et al. 2010). Similar to the requirement of multiple growth factors for tissue regeneration, treatment of a complex disease condition also required temporal administration of different drug to counter the cascade of event that that form the basis for the pathogenesis of the condition. Thus sequential delivery of multiple drugs/growth factors in different temporal profiles would be critical for treatment of complex disease conditions and regeneration of tissues.

2.2 Current research on simultaneous and sequential multiple drug delivery systems

Localized delivery of drug using biodegradable polymer systems has been studied widely and various drug delivery systems have been successfully fabricated for treatment of several disease conditions and regeneration of different types of tissues (Langer and Chasin 1990, Schacht 1990, Jain, Yenet Ayen et al. 2011). There are examples of drug delivery systems capable of releasing small molecule drugs and growth factors. For example widely used small molecule drugs like antibiotics has been delivered using different types of biodegradable polymers (Giamarellos-Bourboulis 2000, Tsourvakas 2000, El-Husseiny, Patel et al. 2011). Similarly in the case of tissue engineering there are different types of delivery systems for local release of growth factors for bone regeneration (Geiger, Li et al. 2003, Saito, Murakami et al. 2005, Ginebra, Traykova et al. 2006). In spite of considerable success at both treatment of a disease and tissue regeneration, more and more research suggests the need for more than one type of drug for a comprehensive treatment of a complex disease condition like the ones that are discussed in the section 2.1 or complete regeneration of a tissue. The multiple drug delivery devices aimed at treatment of complex disease condition and regeneration of tissue are mostly designed for simultaneous delivery of multiple agents (Lynch, de Castilla et al. 1991, Raschke, Wildemann et al. 2002, Nevins, Camelo et al. 2003, Simmons, Alsberg et al. 2004, Dogan, Gumusderelioglu et al. 2005, Peattie, Rieke et al. 2006, Riley, Fuegy et al. 2006, Nillesen, Geutjes et al. 2007, Patel, Young et al. 2008, Chen, Chen et al. 2009, Young, Patel et al. 2009, Borselli, Storrie et al. 2010, Chen, Zhang et al. 2010) rather than sequential delivery.

Poly(lactic-co-glycolic acid) (PLGA) is one of the most commonly used and well characterized biodegradable polymers used for fabrication of various drug delivery systems (Middleton and Tipton 2000, Makadia and Siegel 2011), and many of these multi-drug delivery systems discussed above are in part or completely fabricated using PLGA. PLGA in the form of porous scaffolds has been used for delivery of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) for angiogenesis (Richardson, Peters et al. 2001). PLGA has also been used along with β-tricalcium phosphate in the form of composite scaffolds for controlled dual release of dexamethasone and bovine serum albumin (as a model protein) (Yang, Tang et al. 2011). Different growth factors (IGF-I and TGF-β) have been loaded in PLGA microspheres and embedded in a poly(ethylene oxide)

hydrogel matrix to attain dual drug delivery (Elisseeff, McIntosh et al. 2001). A similar strategy was used by loading gelatin microspheres with VEGF and bone morphogenetic protein 2 (BMP-2) and confining them in a porous poly(propylene fumarate-co-ethylene glycol) hydrogel scaffold for bone regeneration in a critical size defect model (Patel, Young et al. 2008).

Hydrogels have also been used for developing dual drug delivery systems. Alginate hydrogels have been used separately for delivery of BMP-2 and TGF-β_3 for *in vivo* bone formation (Simmons, Alsberg et al. 2004) and in combination with calcium sulphate to deliver both IGF-I and VEGF for muscle regeneration (Borselli, Storrie et al. 2010). Glycidyl methacrylated dextran (Dex-GMA)/gelatin hybrid hydrogel scaffolds with the capability of delivering IGF-I and BMP-2 have been fabricated aimed at periodontal tissue engineering (Chen, Chen et al. 2009). Other than the above mentioned PLGA and hydrogel based systems a cell adhesive scaffold based on poly (2-hydroxyethyl-methacrylate)and poly(L-lysine) has been used for delivery of nerve growth factor (NGF) and neurotrophin-3 (NF-3) to direct axonal growth (Moore, MacSween et al. 2006). There is also a dual delivery system in the form of polylactide coating for delivery of IGF-I and TGF-β to accelerate osteotomy healing (Raschke, Wildemann et al. 2002). Along with the above mentioned microscale dual delivery systems, there are different liposomal based nanoscale delivery system for delivery of multiple drugs for cancer treatment (Chen and Jin 2010, Lee and Nan 2012).

The multi-drug delivery systems which have been discussed till now are mostly the ones that involve simultaneous release of the drugs or growth factors. As mentioned in the section 2.1 all the physiological conditions in our body are a cascade of events. So to treat a condition effectively the drugs must also be delivered in a sequential manner depending on the pathogenesis of the condition and the growth factors must be expressed in the appropriate temporal sequence for successful tissue regeneration. Considering the research done on drug delivery using biodegradable polymers, only a limited amount research has been conducted on sequential drug delivery systems (Chen, Silva et al. 2007, Buket Basmanav, Kose et al. 2008, Jaklenec, Hinckfuss et al. 2008, Kempen, Lu et al. 2009, Tengood, Kovach et al. 2010). As observed in the case of simultaneous multi-drug delivery systems, even in the case sequential drug delivery systems PLGA is the predominantly

used biodegradable polymer. PLGA has been used in the form microsphere-based scaffolds for sequential release of bioactive IGF-I and TGF-β1 (Jaklenec, Hinckfuss et al. 2008), in the form of porous bilayered scaffold to achieve spatiotemporal VEGF and PDGF delivery for blood vessel formation and maturation (Chen, Silva et al. 2007) and in the form of microspheres incorporated into a solid poly(propylene fumarate) (PPF) rod surrounded by a cylindrical gelatin hydrogel for local sequential VEGF and BMP-2 delivery (Kempen, Lu et al. 2009). The sequential release of BMP-2 and BMP-7 has been studied using two different delivery systems both involving PLGA. One system uses PLGA nanoparticles along with Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) nanoparticles embedded in the poly(ε-caprolactone) matrix for sequential release for *in vitro* bone regeneration (Yilgor, Tuzlakoglu et al. 2009, Yilgor, Hasirci et al. 2010), and the other system is based on microspheres of polyelectrolyte complexes of poly(4-vinyl pyridine) (P4VN) and alginic acid loaded with the growth factors BMP-2 and BMP-7 embedded into the scaffolds constructed of PLGA. (Buket Basmanav, Kose et al. 2008). Other examples of delivery systems for sequential drug release include the calcium sulphate nanoparticles incorporated in a double layer collagen membrane (Cao and Bae 2012) or the use of alginate hydrogels for sequential release of VEGF and PDGF (Hao, Silva et al. 2007). Sequential release kinetics of two (gentamicin and BMP-2) or three (gentamicin, IGF-I and BMP-2) bioactive molecules using one component poly (lactic acid) (PLA) coating on k-wires were studied to treat infection and aid bone healing (Strobel, Bormann et al. 2011).

PLGA is a copolymer of glycolic acid and lactic acid linked together by ester linkages. PLGA undergoes bulk degradation resulting in characteristic initial burst release and diffusion of the loaded drug before complete polymer degradation (Makadia and Siegel 2011). Even though sequential release is achieved to a certain extent, clear distinction between the temporal release profiles of different drugs/growth factors is not found in the sequential delivery systems mentioned in this section. For distinct sequential delivery of drugs a surface eroding system capable of releasing drugs only when the polymer erosion occurs would be more suitable, as it would avoid simultaneous diffusion of multiple drugs and aid release of one drug at a time if designed in an appropriate way. Polyanhydrides and poly(orthoesters) are the most common surface eroding polymers used to delivery drugs at a constant rate that is proportional to polymer erosion. Polyanhydrides are copolymers of

aromatic and aliphatic anhydrides that degrade in to non-toxic diacidic monomers. Poly(orthoesters) are hydrophobic surface eroding polymer prepared by tranesterification reaction or by the addition of polyols to diketene acetals (Uhrich, Cannizzaro et al. 1999, Heller, Barr et al. 2002, Kumar, Langer et al. 2002, Jain, Modi et al. 2005).

For designing a device capable of loading and releasing multiple drugs, the polymer system chosen must be compatible with all the drugs that are part of the system, and the processing conditions involved must not affect the bioactivity of any of the drugs. Difficult processing conditions, like high processing temperature and poor solubility of polyanhydrides and poly(orthoesters), makes them a unsuitable for designing complex delivery systems without affecting the bioactivity of the drugs. An association polymer system (CAPP) formed via hydrogen bonds between cellulose acetate phthalate (CAP) and Pluronic F-127 (P) when mixed in an aprotic solvent, is a surface eroding system used for drug delivery (Xu and Lee 1993, Gates, Grad et al. 1994). The CAPP system has been previously used in the form compressed microsphere scaffolds for intermittent and sequential drug delivery of (Raiche and Puleo 2003, Raiche and Puleo 2004, Jeon, Piepgrass et al. 2008).

2.3 Periodontitis and drugs used for its treatment

Periodontitis is inflammatory condition caused by bacterial infection, which results in loss of soft tissue, alveolar bone and ultimately the tooth (Rosen 2001). Periodontitis is the most prevalent inflammatory disease in the world and is the leading cause of tooth loss in adults (Brown, Oliver et al. 1989, Albandar and Kingman 1999). Current methods involve treatment of infection using antibiotics, but in severe cases surgical intervention is required (Chen and Jin 2010). Some of the common treatment methods include mechanical debridement of periodontal pockets along with plaque control to fight the bacterial infection (Etienne 2003). There is no current treatment method available for complete cure of periodontitis (Chen and Jin 2010). Periodontitis is also considered as a risk factor for cardiovascular disease (Beck, Garcia et al. 1996) and preterm low birth weight (Beck, Garcia et al. 1996). Periodontitis being a condition with complex sequential relationship between infection, inflammation and tissue loss, it might require administration of multiple drugs in an appropriate sequence for proper treatment (Jeon, Piepgrass et al. 2008, Cochran

D L. (2003)). Local delivery of drugs has been attempted for treatment of periodontitis (Greenstein and Polson 1998). Vyas et al. reviewed the current research on controlled delivery systems for treatment of periodontitis (Vyas, Sihorkar et al. 2000). All these delivery systems are aimed at the delivery of one type of drug for treatment of one specific part of the periodontitis.

Of the four types of drugs that are required for the complete treatment of periodontitis, the antibiotic is the first drug that has to be administered to fight the bacterial infection. There are different antibiotics, like amoxicillin (Griffiths, Ayob et al. 2011), Moxifloxacin (Ardila, Fernandez et al. 2010), clindamycin, metronidazole, phenoxymethylpenicillin and tetracycline (Slots and Ting 2002, Kulik, Lenkeit et al. 2008) (Eick and Pfister 2004) that have been used against the bacterial infection as a part of the periodontal treatment. Among these amoxicillin, tetracycline and metronidazole were the most effective antibiotics against different types of periodontal bacteria such as *A. actinomycetemcomitans, P. gingivalis* and *P. intermedia/P. nigrescens* (Kulik, Lenkeit et al. 2008). Amoxicillin, doxycycline and Metronidazole have been effectively tested against different strains of *P gingivalis* (Larsen 2002). Local delivery of antibiotics has been attempted for the treatment of periodontal infection (Bernie , Schwach-Abdellaoui, Vivien-Castioni et al. 2000, Etienne 2003). Studies have shown that biofilm-associated *P gingivalis* might be resistant against metronidazole concentration that is being attained by systemic administration (Wright, Ellen et al. 1997). Metronidazole has been delivered locally for periodontal treatment in the form of gel (Sato, Fonseca et al. 2008), electrospun poly(L-lactide-co-D/L-lactide) fibers (Reise, Wyrwa et al. 2012) and mucoadhesive buccal formulation (Perioli, Ambrogi et al. 2004).

The other type of drug that is administered for treatment of periodontitis is an anti-inflammatory drug to counter the inflammatory response that was caused due to bacterial infection (Van Dyke 2008). Non-steroidal anti-inflammatory drugs (NSAIDs) have been one of the common class of drugs used in periodontal treatment (Williams, Jeffcoat et al. 1984, Offenbacher, Williams et al. 1992, Howell and Williams 1993, Salvi and Lang 2005). Studies have shown that NSAIDs also have a positive effect by altering the human alveolar bone loss progression (Lynch, Williams et al. 1989, Howell, Jeffcoat et al. 1991, Dionne and Berthold 2001). Some of the commonly used NSAIDs for treating periodontitis include

flurbiprofen (Jeffcot, Williams et al. 1986, Lynch, Williams et al. 1989), ketoprofen (Reed, Smith et al. 1997), indomethacin (Williams, Offenbacher et al. 1988) and naproxen (Howell, Jeffcoat et al. 1991). Local delivery of these drugs have been attempted to treat periodontitis using acrylic bone cement as the delivery vehicle (Corry and Moran 1998) or delivery of these drugs using microspheres (Paquette, Oringer et al. 2003) or cellulose acetate films (Cetin, Buduneli et al. 2004). Flurbiprofen has been administered locally in topical form as an adjunct to non-surgical management of periodontal disease (Heasman, Benn et al. 1993). Similarly topical administration of ketoprofen in the form of cream (Howell, Martuscelli et al. 1996) or gel (Lawrence, Paquette et al. 1998) have shown positive results towards potential inhibition of disease progression (Paquette, Fiorellini et al. 1997, Paquette, Lawrence et al. 2000).

The loss of alveolar bone caused because of the adverse inflammatory response (Cochran 2008) is treated using anti-resorptive agents. Bisphosponates are a class of anti-resorptive drugs, which have been used for the treatment of periodontitis (Tenenbaum, Shelemay et al. 2002, Lane, Armitage et al. 2005, Shinoda and Takeyama 2006, Jeffcoat, Cizza et al. 2007, Badran, Kraehenmann et al. 2009). Residronate administered at an appropriate dosage has shown to inhibit bone resorption in periodontitis (Cetinkaya, Keles et al. 2008). Studies have also shown that the topical administration of olpadronate has effectively prevented bone loss caused by periodontitis (Goya, Paez et al. 2006). Local administration of bisphosphonate such as alendronate using gelatin sponges has also been shown to reduce bone loss in periodontal procedures such as mucoperiosteal flap surgery (Reddy, Weatherford et al. 1995, Yaffe, Fine et al. 1995, Yaffe, Iztkovich et al. 1997, Binderman, Adut et al. 2000, Kaynak, Meffert et al. 2000). Tetracycline has significant anti-matrix metalloproteinase activity and also inhibits osteoclast development, structure, and function, thereby helps prevent bone resorption (Vernillo and Rifkin 1998) Tetracycline has also been shown to be particularly effective against alveolar bone loss associated with periodontitis (Ramamurthy, Rifkin et al. 2002). Tetracycline when applied locally along with bisphosphonate reduced alveolar bone loss (Yaffe, Herman et al. 2003). Doxycycline, a type of tetracycline with antibacterial properties, when administered in subantimicrobial dose has been shown to improve efficacy of scaling and root planning along with having a positive effect on the management of severe, generalized, chronic

periodontitis (Caton, Ciancio et al. 2000, Novak, Johns et al. 2002). Doxycycline when delivered locally was equally effective as scaling and root planning (Garrett, Johnson et al. 1999), and when combined with a NSAID had enhanced effect in inhibition of matrix metalloproteinase for treatment in chronic periodontitis patients (Lee, Ciancio et al. 2004).

The final stage of periodontitis treatment would involve the regeneration of the lost tissue, which would include the regeneration of the alveolar bone. There are different growth factors that have been locally delivered using biodegradable polymers like PLGA, PLA and poly(caprolactone) (PCL) for regeneration of bone (Tezcaner and Keskin 2011). One of the most common growth factor used for bone regeneration is BMP (Haidar, Hamdy et al. 2009, Brown, Li et al. 2011). Different growth factors including the BMPs, have been used for alveolar bone regeneration and reconstruction (Graves, Kang et al. 1994, Sigurdsson, Lee et al. 1995, Giannobile 1996, Howell, Martuscelli et al. 1996, Toriumi, O'Grady et al. 1999, Raja, Byakod et al. 2009). Specifically BMP-2 has been used for periodontal reconstruction with significantly enhanced regeneration (Sigurdsson, Lee et al. 1995, Wikesjo, Guglielmoni et al. 1999, Selvig, Sorensen et al. 2002, Saito, Saito et al. 2003). BMP-2 has been delivered using gelatin based carrier system (Talwar, Di Silvio et al. 2001) or in the form of gene therapy for periodontal regeneration (Jin, Anusaksathien et al. 2003, Dunn, Jin et al. 2005, Chen, Chen et al. 2008, Lutz, Park et al. 2008). Other than BMP-2, platelet-derived growth factor has been studies for the purpose of periodontal regeneration (Giannobile, Lee et al. 2001, Nevins, Camelo et al. 2003). Combination of growth factors have also been used for periodontal regeneration, such as the application of human osteogenic protein-1 and BMP-2 (Ripamonti, Crooks et al. 2001) and the use of PDGF and IGF to enhance bone formation (Lynch, Williams et al. 1989, Lynch, de Castilla et al. 1991). Apart from the growth factors used for periodontal regeneration, simvastatin a small molecule drug has been shown to promote osteoblast viability and differentiation via membrane bound BMP-2 pathway (Chen and Jin 2010). Simvastatin has also been studied for its potential as osteogenic agent in treatment of periodontitis (Singh, Dodwad et al. , Yazawa, Zimmermann et al. 2005, Chen and Jin 2010). Simvastatin has been shown to promote osteoblast viability and differentiation via membrane bound BMP-2 pathway Studies have shown that the administration of simvastatin had a positive effect on the periodontitis-associated bone loss in rat model (Vaziri, Naserhojjati-Roodsari et al. 2007).

Local delivery of simvastatin in the form of injection (Morris, Lee et al. 2008) and gels (Thylin, McConnell et al. 2002) resulted in improved treatment outcome both in rat model (Stein, Lee et al. 2005, Seto, Ohba et al. 2008) and human clinical trials (Pradeep and Thorat 2010).

2.4 Significance

Published research on simultaneous and sequential release of drugs mostly involves only delivery of two types of drugs or growth factors. This research focuses on sequential release of four different drugs at different rates and doses to create a versatile system. Most of the multiple drug delivery systems are formed using more than one type of polymer and use combinations of microspheres or nanoparticles embedded in scaffold or matrix in a form of complex architecture resulting in a relatively difficult fabrication process. The multilayered device used in this research would be primarily formed using CAPP association polymer in the form of drug loaded films with a relatively simpler fabrication process. Even though the device is capable of delivering different types of drugs, this research is aimed at designing a device capable delivering four drugs in a predetermined sequence for stepwise treatment of periodontitis. Periodontitis was chosen as a representative example of a condition that may benefit by sequential drug delivery. The focus of this research is on developing a device specifically for sequential release of antibiotics, anti-inflammatory, anti-resorptive and osteogenic drugs for periodontal treatment by elimination of infections and inflammation, prevention of bone resorption and augmentation of alveolar bone growth.

The drugs that were selected to be loaded in this device have already been successfully used separately for the treatment of a specific stage of periodontitis as discussed in section 2.3. The metronidazole (antibiotic) and ketoprofen (anti-inflammatory) used as a part of this device are the currently being in use for periodontitis treatment. The third drug that is a part of this device, doxycycline, is a tetracycline with both anti-resorptive and antibiotic properties. This will help prevent bone resorption and also the antibiotic property of this drug will provide of protection, along with metronidazole against infection during the course of repair and regeneration. The final osteogenic drug selected is simvastatin. Simvastatin, being a small molecule drug it is

relatively more robust during fabrication process than the growth factors that have been used for periodontal regeneration. This device will deliver these drugs in a sequential order to form an all-encompassing treatment for periodontitis. The device can be altered by inclusion of poly(sebasic acid) (PSA) layer or by changing the ratio of CAP:Pluronic to achieve different erosion times and release profiles as required by the severity of the condition. The amount of drug loaded and released from the CAPP films can also be altered based on the dose requirement, which would be of critical importance for effective treatment. The final part of this research included the study *in vivo* drug release profiles from these multiple drug delivery devices.

Chapter 3 Bioerodible System for Sequential Release of Multiple Drugs

3.1 Introduction

Several conditions, such as severe bacterial infection,(Dowling 1957) periodontitis,(2001) and traumatic bone loss along with infection,(Younger, Duncan et al. 1998) require repeated administration of a drug or administration of more than one drug for efficacious treatment. As reviewed by Chen et al., delivery of multiple growth factors is also important for tissue engineering.(Chen, Zhang et al. 2010) Thus, this research was directed at developing a bioerodible system capable of delivering one or more types of drug in a predetermined temporal sequence, which could be helpful for treatment of different stages of complex diseases and also in tissue engineering.

Polyanhydrides and polyorthoesters are two common classes of surface-eroding polymers employed for controlled delivery of drugs for a variety of purposes, including antimicrobial, anti-inflammatory, analgesic, cancer, and ocular applications.(Uhrich, Cannizzaro et al. 1999, Heller, Barr et al. 2002, Kumar, Langer et al. 2002, Jain, Modi et al. 2005) Surface-eroding polymers provide a constant rate of drug release that is directly proportional to polymer erosion.(Jain, Modi et al. 2005) As such, they provide highly controllable and reproducible drug release profiles(Uhrich, Cannizzaro et al. 1999) that would be useful for designing multiple drug delivery systems. Ease of processing is an important consideration for designing and developing a versatile drug delivery system capable of delivering more than one type of drug, but the high processing temperature and poor solubility in organic solvents cause difficulty in fabrication of some polyanhydrides and polyorthoesters into dosage forms.(Gopferich and Tessmar 2002, Heller, Barr et al. 2002)

An alternative, but less well known, surface-eroding system is composed of cellulose acetate phthalate (CAP) and Pluronic F-127 (P). When mixed in an aprotic solvent, the polymers associate via hydrogen bonds.(Xu and Lee 1993) The properties of the CAPP system, such as ease in fabrication and drug loading, make it a suitable candidate for designing a surface-eroding multiple drug delivery system. The CAPP association polymer system has already been studied for the release of a single drug.(Xu and Lee 1993, Gates, Grad et al. 1994) For example, Xu et al. demonstrated the effect of the CAP to

Pluronic ratio on erosion rate and drug release.(Xu and Lee 1993) The CAPP association polymer has also been used in the form of consolidated microspheres for the release of protein.(Raiche and Puleo 2003) Jeon et al. fabricated CAPP microsphere-based devices for intermittent release of simvastatin and showed positive results for osteoblast responses and bone formation *in vitro* and *in vivo*, respectively.(Jeon, Thomas et al. 2007, Jeon, Piepgrass et al. 2008) The same group also studied intermittent release of two different drugs using CAPP microsphere-based devices.(Jeon, Piepgrass et al. 2008) There is less research, however, toward delivery of more than two drugs or biomolecules in a predetermined temporal sequence.

In the present studies, different small molecule drugs, such as metronidazole (antibiotic), doxycycline (antibiotic/anti-resorptive), ketoprofen (anti-inflammatory) and simvastatin (hypolipidemic/osteogenic) along with a model protein (lysozyme) were loaded in CAPP films. After evaluating individual layers, the morphology of multilayered devices and subsequent intermittent and sequential release profiles were measured. To determine effects of encapsulation and release on bioactivity, enzymatic activity of the released model protein was determined.

3.2. Materials & Methods

3.2.1 Fabrication of CAPP films

CAPP films were fabricated by solvent casting. CAP (Sigma, St. Louis, MO) and Pluronic F-127 (Sigma) were mixed in the weight ratio of 70:30, respectively, and dissolved in acetone to obtain an 8% polymer solution. Either 2.5 or 5 wt% of drug was added to the acetone-polymer (CAPP) solution and mixed thoroughly until the drug was completely dissolved, except for the case of the model protein, which did not completely dissolve. The drug-polymer solution was poured in a Teflon dish and stored at 4ºC for 24 hours for solvent evaporation to take place. Blank CAPP films were prepared in the same way but without the addition of drugs. For the present study, CAPP films were loaded with metronidazole (Sigma), doxycycline (Sigma), ketoprofen (Sigma), lysozyme (Sigma), or simvastatin (Haorui Pharma-Chem, Inc., Edison, NJ). Samples with 6 mm diameter and 0.5 mm thickness were punched from the CAPP films for further study.

Multilayered CAPP films were fabricated to obtain intermittent release of the same drug or sequential release of more than one drug. Figure 3.1 shows a schematic representation of the fabrication process. The drug-loaded and blank CAPP films were arranged in the desired sequence and then bonded together by compressing them after 5 μL of acetone were applied between the layers. For intermittent release of drugs, four-layered CAPP devices were prepared with alternating layers of blank and metronidazole-loaded films. For achieving sequential release of more than one type of drug, three-layered CAPP devices were fabricated using metronidazole- and ketoprofen-loaded CAPP films with blank films between the drug-containing layers. The stacked CAPP films were inserted into a 6 mm diameter polystyrene well, which acted as in impermeable backing to enable unidirectional polymer erosion and drug release. A similar procedure was followed to fabricate multilayered devices loaded with simvastatin and doxycycline.

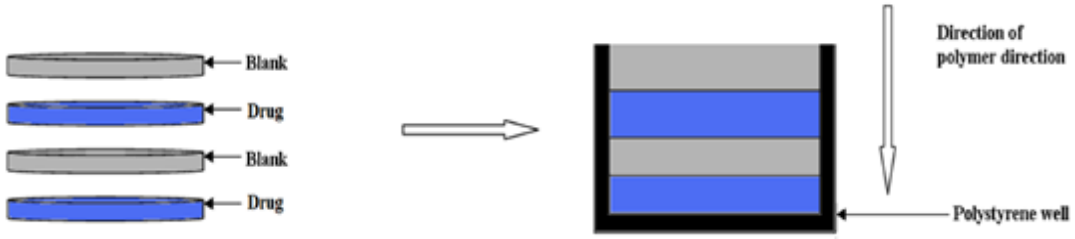


Figure 3.1: Schematic representation of the process for fabricating multilayered CAPP devices.

3.2.2 Morphological characterization of CAPP films

Scanning electron microscopy (SEM)

SEM imaging was used to study the overall morphology and interfaces of the blank and drug-loaded CAPP films that form the device. For this purpose, four-layered devices were fabricated with alternating metronidazole-loaded and blank films. The CAPP films were freeze-fractured, and the cross-section was sputter-coated with platinum and observed using an S-3200-N Hitachi instrument.

Fluorescence imaging

To analyze the spatial distribution of drug following device fabrication, a fluorescent molecule was incorporated into multilayered films. Fluorescein (Sigma) was loaded in CAPP films at 0.16 wt%, and multilayered CAPP films were fabricated with alternating layers of fluorescein-loaded and blank films. Thin (5 µm) cross-sections of the multilayered CAPP films were cut with a microtome and observed under epifluorescence (Olympus IX51). To determine the effect of aging on interlayer diffusion of fluorescein, samples were incubated at 37°C for 6 days followed by sectioning and microscopic analysis. Line profiling of the fluorescent microscopic images was conducted using ImageJ software.

3.2.3 Drug release from single and multilayered CAPP films

In vitro release studies were conducted for single-layered CAPP films by eroding the materials in 4 mL of phosphate-buffered saline (PBS), pH 7.4, at 37ºC on an orbital shaker. Release supernatant was collected every one hour and replaced with fresh PBS. Blank CAPP films of the same dimensions were used as controls. Multilayered devices

were eroded in either 2 or 4 mL of PBS to study the effect of sink volume on device erosion and drug release. For multilayered devices, release supernatants were collected approximately every 8-10 hours and replaced with fresh PBS. Mass loss studies were conducted by measuring the remaining mass of the multilayered CAPP at regular intervals during its course of erosion in 2 ml of PBS. Three-layered blank devices were used as controls for the release and erosion studies. Because lysozyme loaded in the films did not dissolve completely, protein particles were distributed in the CAPP films. To determine whether the heterogeneous distribution affected release, the lysozyme-loaded films were tested in two orientations (protein side up and protein side down) within the polystyrene well.

Supernatants were analyzed using UV spectroscopy (Powerwave HT, Biotek) to determine the concentration of metronidazole (318 nm) and doxycycline (350 nm). High performance liquid chromatography (HPLC; Shimadu Prominence) was used to measure the concentration of ketoprofen (mobile phase of acetonitrile (60):trifluroacetic acid (TFA) buffer (40); UV detection at 260 nm) and simvastatin (mobile phase of acetonitrile (70):TFA buffer (30); UV detection at 240 nm). The BCA protein assay (Pierce, Rockford, IL) was used to quantify the concentration of lysozyme.

3.2.4 Mathematical modelling

Release profiles for drugs released from the CAPP system were evaluated using Hopfenberg's model for controlled release from erodible slabs (Equation 1):

$$\frac{M_t}{M_\infty} = 1 - \left[1 - \frac{k_o t}{C_o a}\right] \quad (1)$$

where *Mt* is the amount of drug released (mg) at time t (hours), $M\infty$ the total amount of drug released from the device (mg), *ko* the erosion constant (mg/hr/mm2), *Co* initial concentration of the drug in the device (mg/mm3), *a* the half thickness of the slab, and n=1 for a slab.(Hopfenberg H 1976) Based on the conditions provided for the model, CAPP devices were considered erodible slabs. Furthermore, only one side of the slab was exposed for polymer erosion and drug release due to the presence of the polystyrene well. To accommodate this condition of unidirectional erosion and release, the term *a* (half the thickness of the slab) was replaced with *2a* (total thickness of the slab in mm) in equation

(1). The predicted release profiles were compared with the experimentally determined cumulative release profiles.

3.2.5 Bioactivity of the released protein

Lysozyme bioactivity was measured by its ability to lyse cell walls of *Micrococcus lysodeikticus* (Sigma).(Ghaderi and Carlfors 1997, Jiang, Hu et al. 2005) Lysozyme release supernatant or standard dilutions of lysozyme in PBS were added to 0.5 mg/mL of *M. lysodeikticus*, and the absorbance at 450 nm was measured at 0 and 10 minutes. The observed and expected (obtained from the standard curve) absorbances were compared to determine the relative bioactivity of released lysozyme.

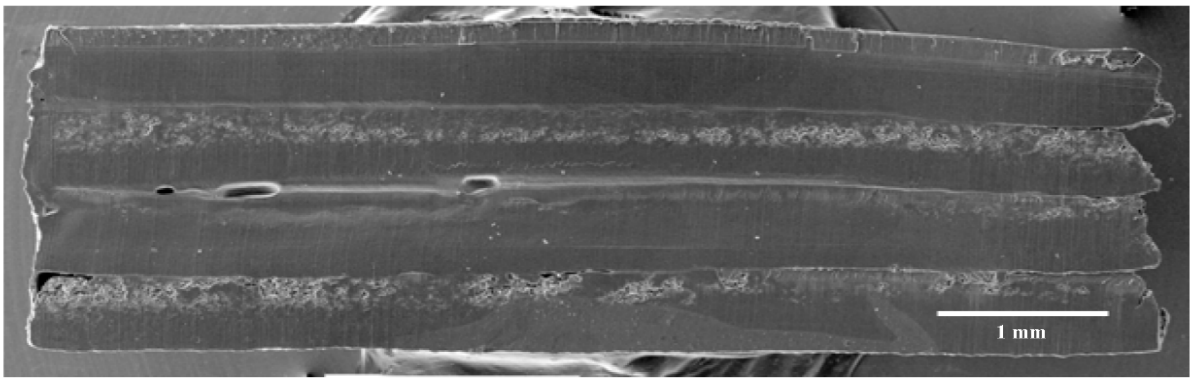
3.2.6 Statistical analysis

Experimental data were analyzed for statistical significance by the Student's t-test using InStat (GraphPad Software, Inc., La Jolla, CA). Slopes of the release profiles for different drugs as well as those obtained from mathematical modeling were analyzed by linear regression using Graphpad Prism software.

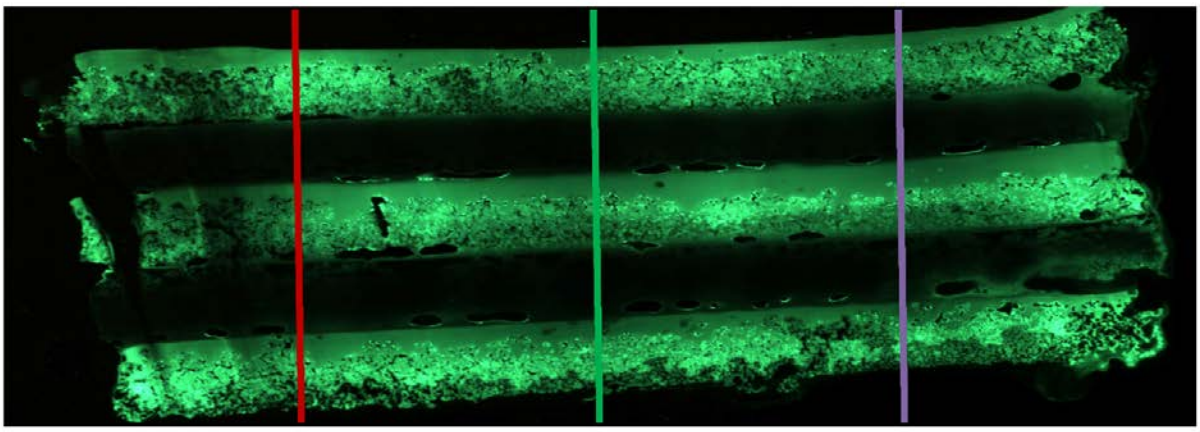
3.3 Results & Discussion

3.3.1 Morphological characterization

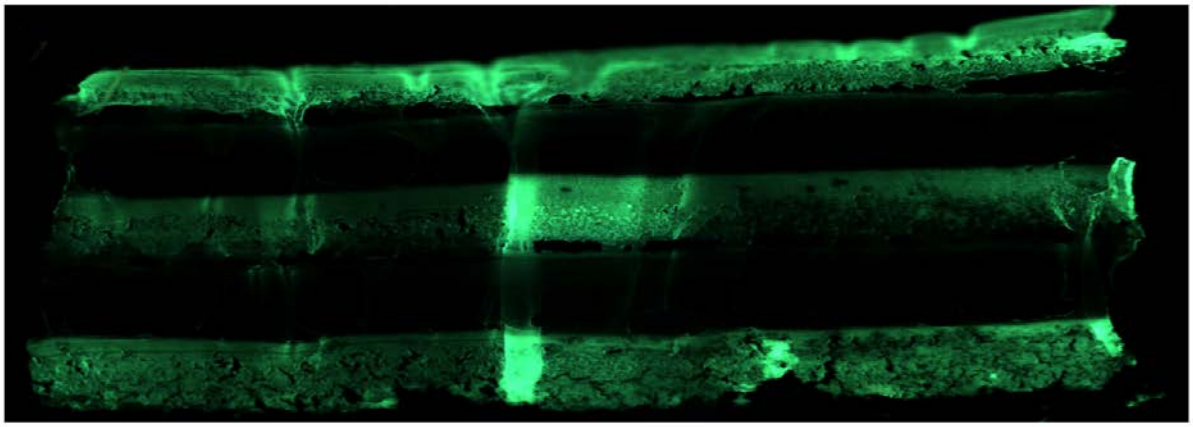
SEM showed clear demarcation between the alternating layers of drug-loaded and blank CAPP films along with some interlayer voids and defects that were likely created during multilayered fabrication (Figure 3.2 A). Figure 3.2 B shows the cross-section of a multilayered CAPP film with alternating layers of fluorescein-loaded and blank CAPP films visualized by fluorescence microscopy. Heterogeneity in distribution was observed on the top/side of the CAPP layer where it was attached to another CAPP layer. Figure 3.2 C shows a cross-section of a sample after 6 days of incubation at 37°C. Both fluorescence images showed that layers loaded with fluorescein were distinctly separate from the blank CAPP layers. Line profiling quantitatively confirmed the distinction between the fluorescein-loaded and blank CAPP films. Line profiles obtained at three different sections of the multilayered device showed clear separation in brightness between layers.



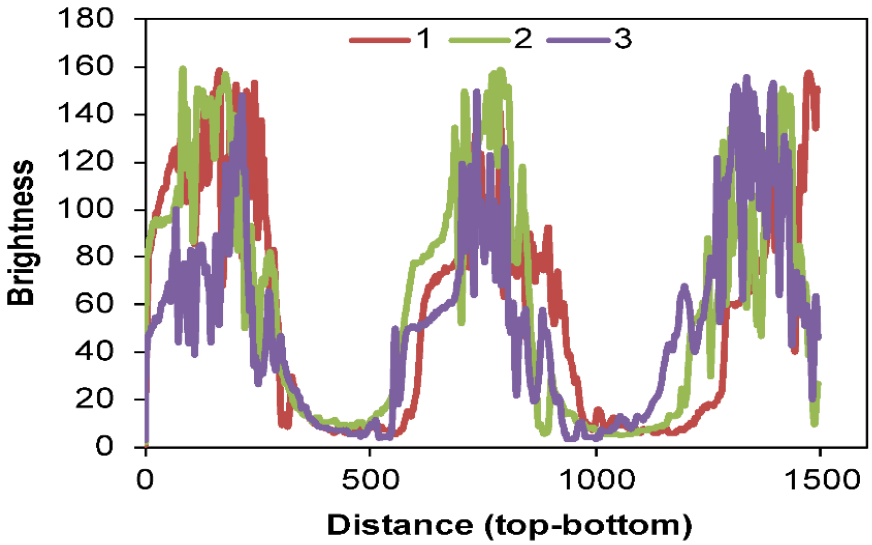
(A)



(B)



(C)

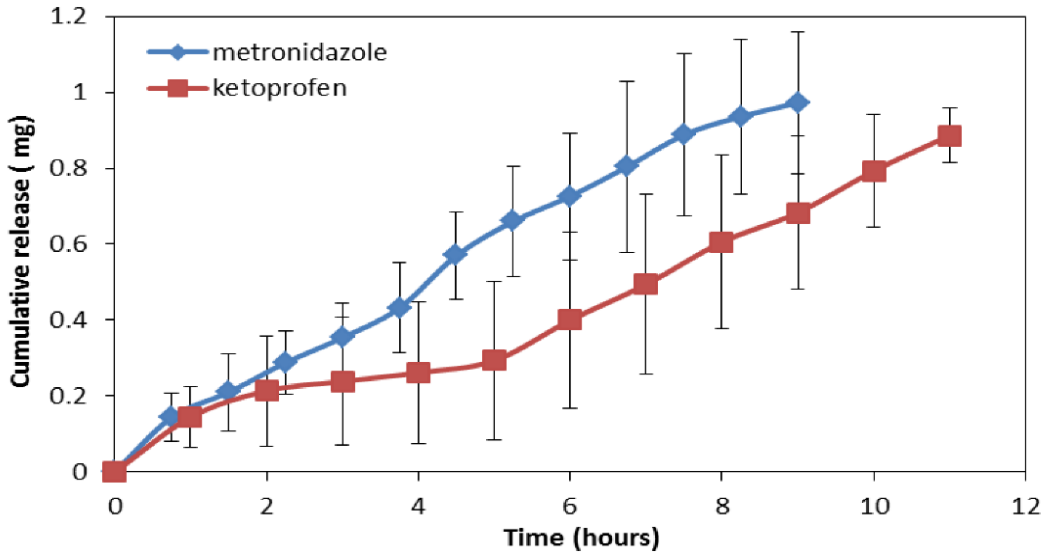


(D)

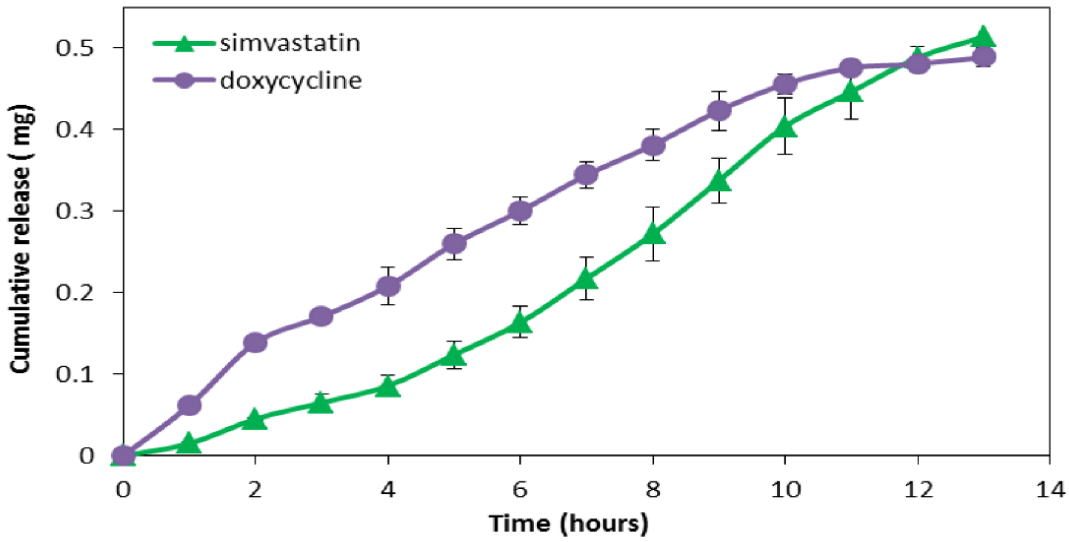
Figure 3.2: Morphology of multilayered CAPP devices. (A) SEM image of the cross-section showing four CAPP films attached to each other. Fluorescent images of multilayered CAPP devices with alternating fluorescein-loaded and blank films obtained (B) one day after fabrication and (C) after 6 days of incubation at 37ºC. (D) Line profiles showing the distinct difference between the fluorescein-loaded and blank layers at different locations (shown in Figure 3.2 B).

3.3.2 Single layer drug release profiles

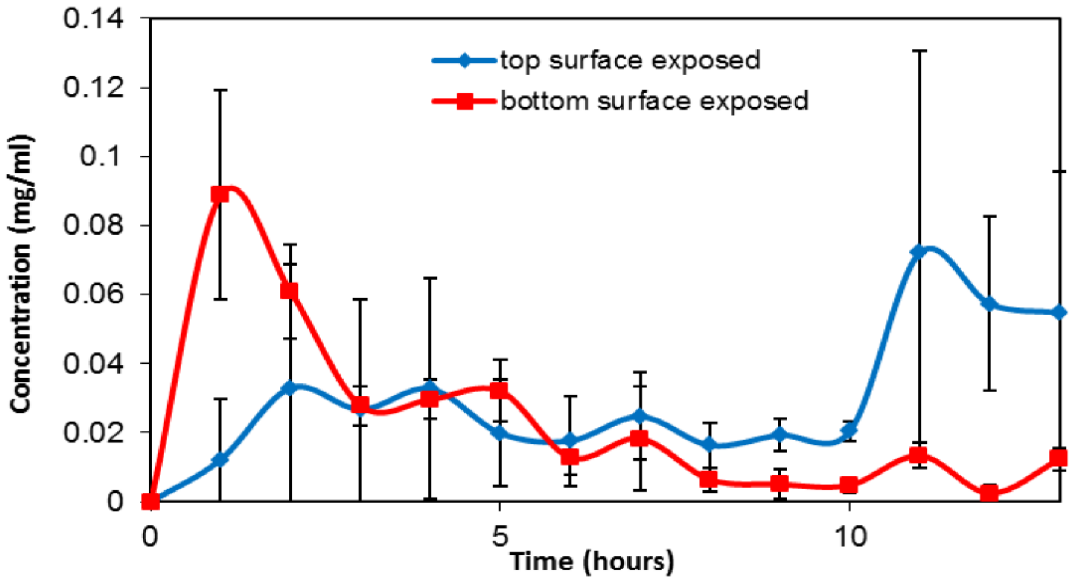
Release of individual drugs (metronidazole, ketoprofen, simvastatin, doxycycline, and lysozyme) from a single layer of CAPP showed sustained release of the drug during the course of erosion (8-10 hours) reflecting near zero-order kinetics (Figure 3.3 A and 3.3 B). The total amount of drug released from the CAPP films corresponded to the amount of drug loaded in the CAPP films. For example, 5 wt% of metronidazole and ketoprofen was loaded in the CAPP films, which resulted in 1 mg of drug in each sample. The cumulative release profile showed that, on average, 97% of the metronidazole and 89% of ketoprofen loaded in the CAPP films were detected (Figure 3.3 A). The loading of simvastatin and doxycycline was 2.5 wt%, resulting in 0.5 mg of drug present in each sample. The average percentages of simvastatin and doxycycline released were 100% and 98%, respectively (Figure 3.3 B). Slopes of the release profiles for different small molecule drugs released from single-layered CAPP films were statistically similar. The release profiles of lysozyme-loaded CAPP films, however, differed from those of the other drugs (Figure 3C). Approximately 60% of the protein was released either during the first 4 hours of film erosion or during the final 4 hours, depending on which surface of lysozyme films was exposed to PBS.


metronidazole
ketoprofen
Cumulative release (mg)
Time (hours)
0
0.2
0.4
0.6
0.8
1
1.2
0
2
4
6
8
10
12

(A)


simvastatin
doxycycline
Cumulative release (mg)
Time (hours)
0
0.1
0.2
0.3
0.4
0.5
0
2
4
6
8
10
12
14

(B)



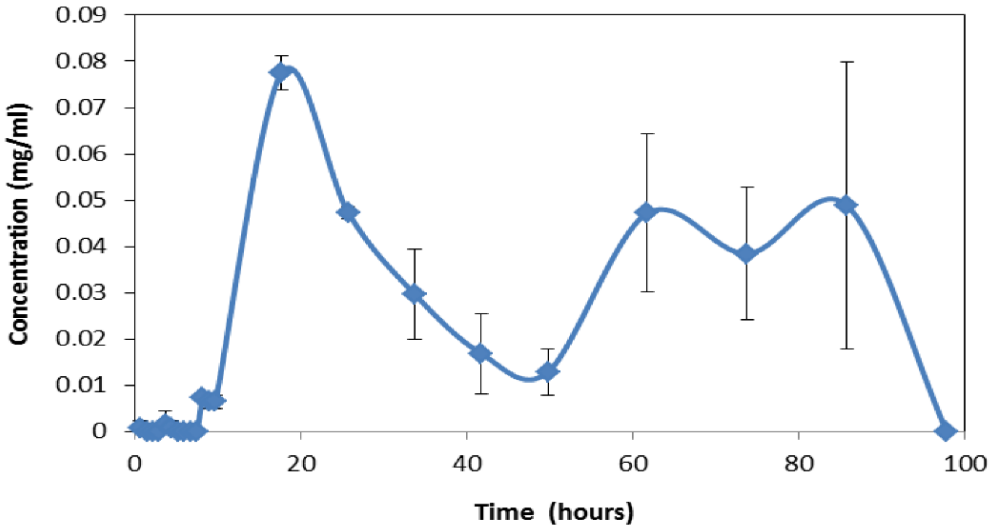
(C)

Figure 3.3: Profiles showing release of drugs from CAPP films. (A) Cumulative release of metronidazole and ketoprofen (5 wt% loading). (B) Cumulative release of doxycycline and simvastatin (2.5 wt% loading). (C) Instantaneous release of lysozyme from CAPP films with top or bottom surface exposed to PBS. Data are mean ± standard deviation (n=3).

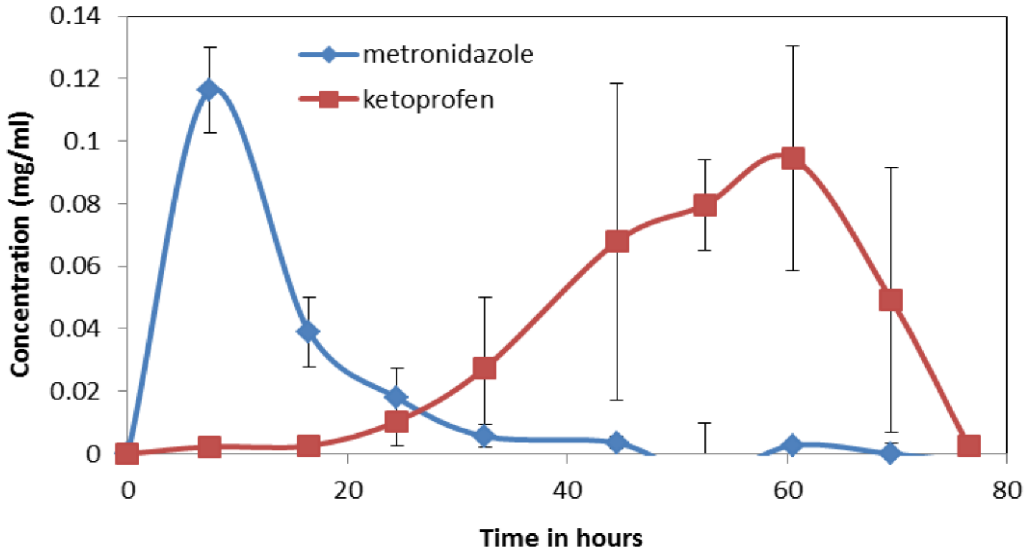
3.3.3 Intermittent and sequential drug release profiles

Polymer erosion of multilayered (four-layered) CAPP devices with alternating metronidazole-loaded and blank layers resulted in intermittent release of the same drug (Figure 3.4 A). This release profile showed no release of drug during the initial stages of erosion (first 10 hours) due to the presence of blank layer on top, followed by release of metronidazole from the second CAPP layer (approximately 10-40 hours). The third (blank) layer delayed release of metronidazole from the fourth layer, while metronidazole from the final (bottom) layer of the device was released during the last stages (last 40 hours) of erosion.

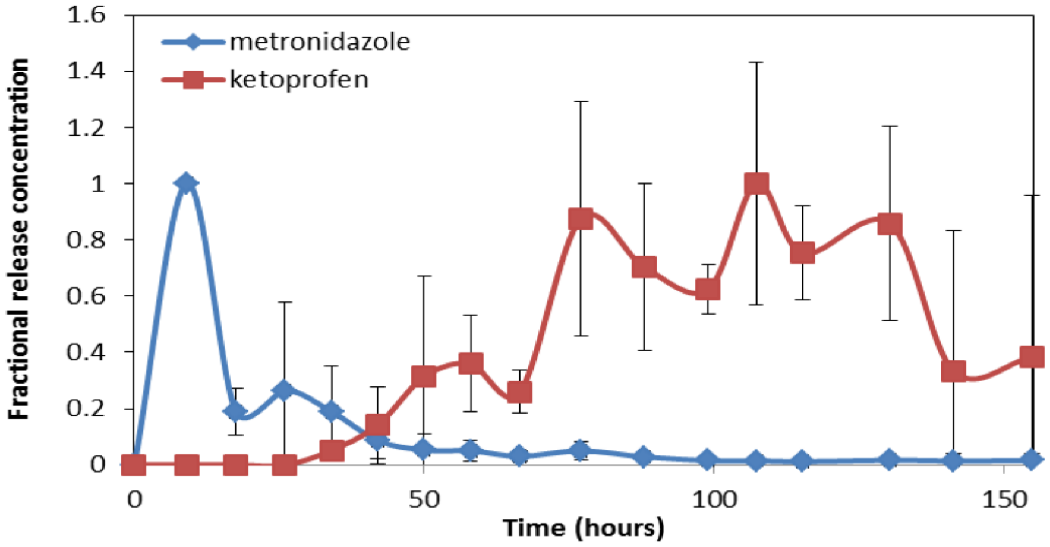
Figure 3.4 B shows release profiles for three-layered devices with metronidazole- and ketoprofen-loaded layers separated by an intermediate blank film eroded in 4 mL of PBS. Based on design of the device, metronidazole was released during the first 20-25 hours of device erosion. The blank layer delayed the next phase of release, which involved release of ketoprofen during the final stages of device erosion (last 40-50 hours). When the same type of device was eroded in 2 mL of PBS, the total erosion time was around 155 hours compared to only 77 hours observed for 4 mL PBS (Figure 3.4 C). Even though the device eroded more slowly when the amount of medium (PBS) was reduced, sequential drug release was still achieved. Metronidazole was released during the first 40 hours of device erosion followed by a small delay in the release of ketoprofen due to the presence of the blank layer; ketoprofen was again released during the last 100 hours of the device erosion.


0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Concentration (mg/ml)
0
20
40
60
80
100
Time (hours)

(A)


0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Concentration (mg/ml)
metronidazole
ketoprofen
0
20
40
60
80
Time in hours

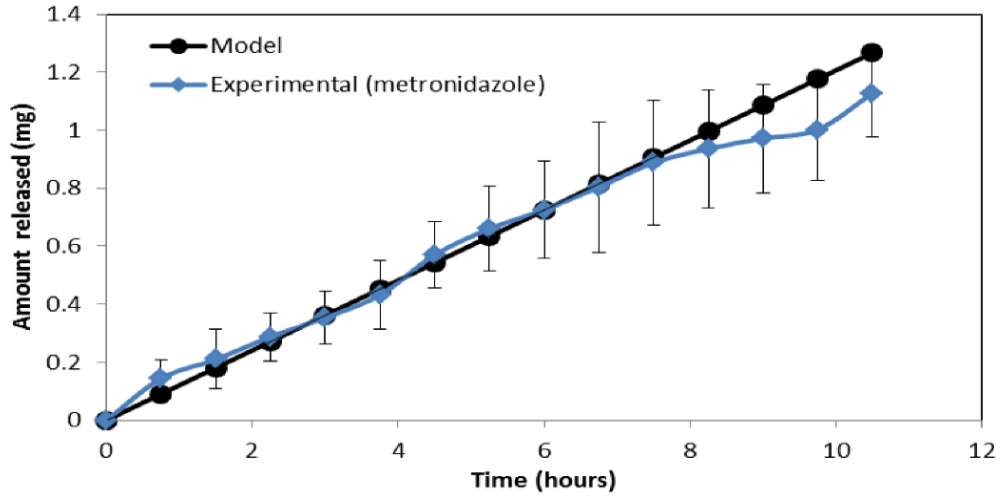
(B)



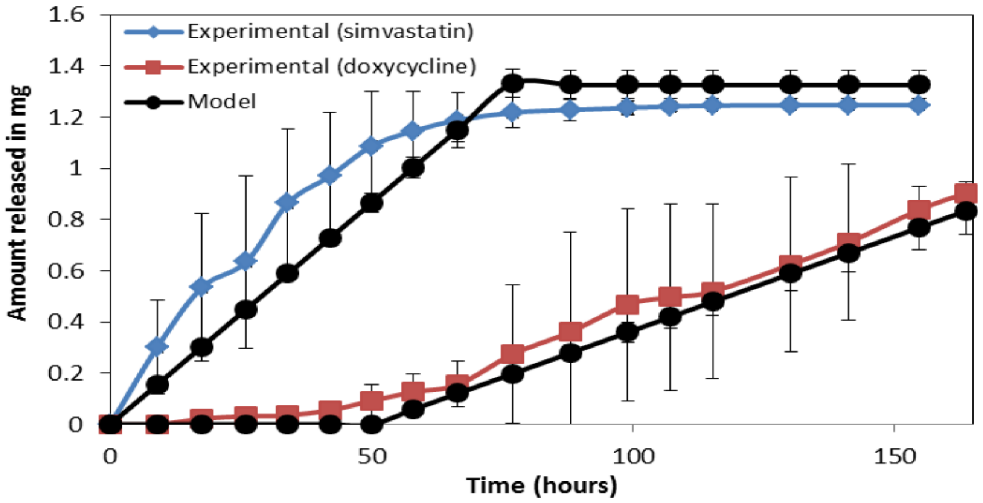
(C)

Figure 3.4: Instantaneous drug release profiles for multilayered CAPP devices. (A) Intermittent release of metronidazole (blank-metronidazole-blank-metronidazole) during erosion in 4 mL of PBS. Sequential release of metronidazole followed by ketoprofen (metronidazole-blank-ketoprofen) during erosion in (B) 4 mL or (C) 2 mL of PBS. Data are mean ± standard deviation (n=3).

Figure 3.5 A shows both empirical and predicted release of one drug (metronidazole) from a single layer CAPP film. As for films containing metronidazole and ketoprofen, multilayered devices with simvastatin and doxycycline eroded in 2 mL of PBS also followed the same sequential release pattern, with a total erosion time of approximately 160 hours (Figure 3.5 B). Simvastatin was released significantly faster during the first 50 hours of device erosion than doxycycline was released during the last 100 hours ($p<0.05$), but there were no significant differences between the experimentally measured slopes and those predicted by mathematical modeling.



(A)



(B)

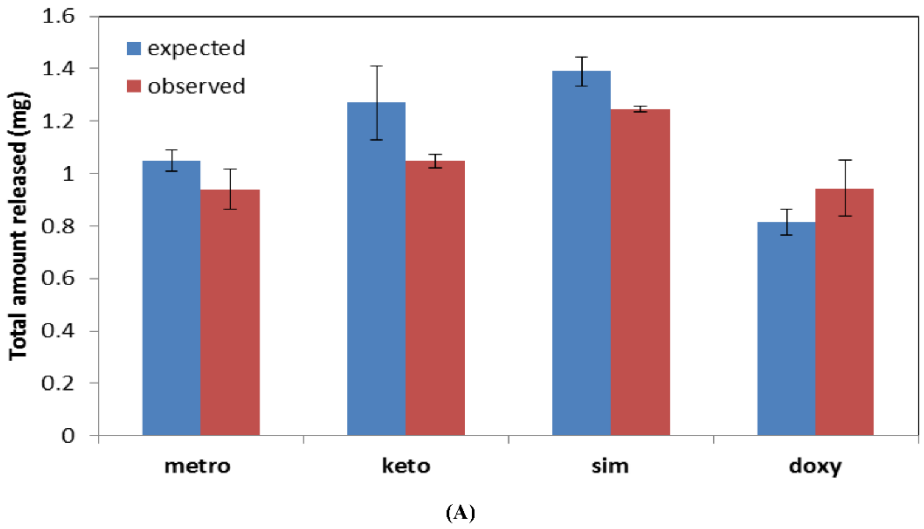
Figure 3.5: Comparison of observed and mathematically predicted cumulative release profiles for (A) metronidazole in a single layer CAPP film and (B) simvastatin followed by doxycycline in a three-layered film. Data are mean ± standard deviation (n=3).

3.3.4 Loading and release efficiency

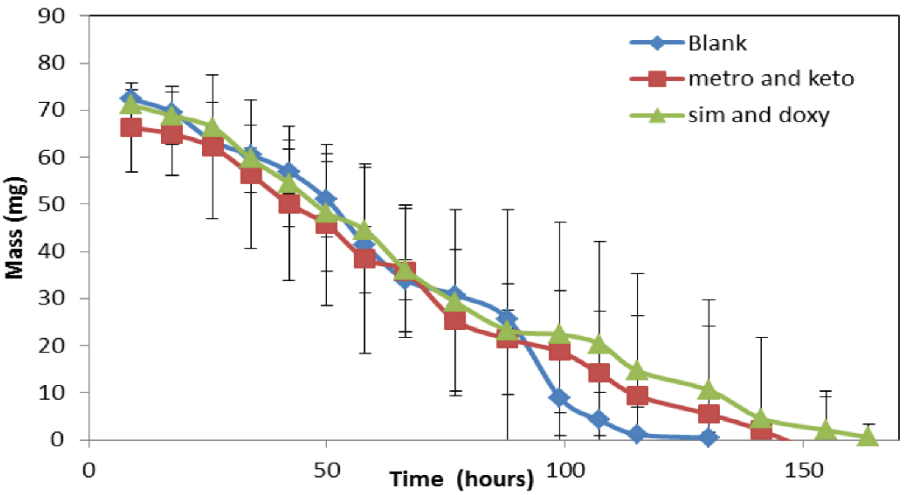
In general, 96.5% of the small molecule drugs loaded into CAPP films was released, irrespective of the type or wt% of the drug (Figure 3.6 A). There was no statistically significant difference between the observed and expected amount of metronidazole and doxycycline loaded and released from the CAPP multilayer devices. In the case of ketoprofen, 83% of the expected amount was released, and in the case of simvastatin 90% of the expected amount was released.

3.3.5 Mass loss profiles

Figure 3.6 B shows mass loss profiles for three-layered devices eroded in 2 mL of PBS. The profiles presented are for blank, sequential metronidazole and ketoprofen, and sequential simvastatin and doxycycline films. Both the blank devices and the drug-loaded devices eroded with linear mass loss profile characteristic of a zero-order system. Neither loading nor the type of drug incorporated into the films had a significant effect on the erosion rate.



(A)



(B)

Figure 3.6: (A) Observed and expected amounts of drugs released from multilayered CAPP devices. (B) Mass loss profiles for multilayered devices with blank layers (blank), layers loaded with metronidazole and ketoprofen (metro and keto), or layers loaded with simvastatin and doxycycline (sim and doxy) degraded in 2 mL of PBS.

3.3.6 Bioactivity of released protein

Figure 3.7 shows the bioactivity of lysozyme in release supernatants during the final 6 hours (time points when lysozyme release occurred) of film erosion. Results showed that, on an average, lysozyme released from the CAPP films retained 57% of the expected bioactivity.

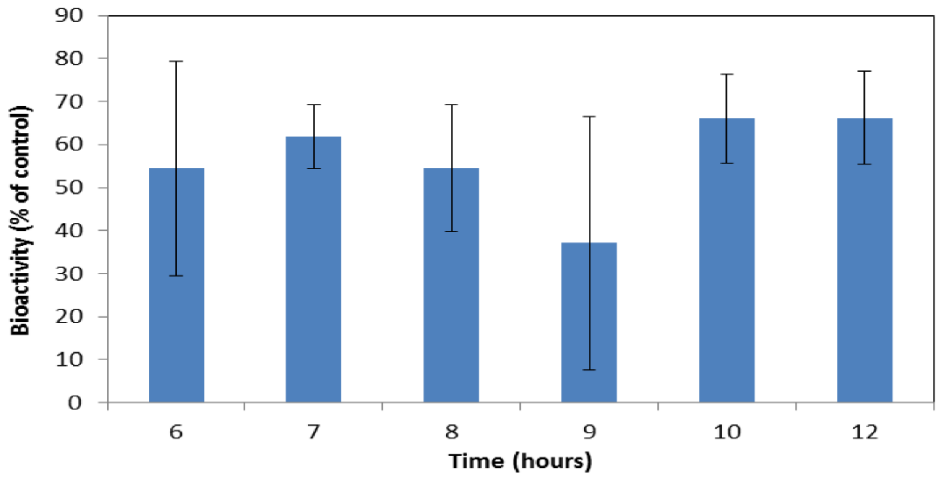


Figure 3.7: Retention of lysozyme bioactivity following release from CAPP films. Data are mean ± standard deviation (n=3).

3.4 Discussion

The main aim of this research was to develop a CAPP film-based system that will serve as a platform for delivery of different types of drugs, suitable for treatment of a broad range of disease conditions. These multilayered devices may also be adapted for delivery of more than one type of biomolecule for tissue engineering applications.

The two polymers used for the association polymer, CAP and Pluronic F-127, form intermolecular hydrogen bonds in aprotic solvents, such as acetone, used during fabrication. In this case, carboxylic acid groups in CAP act as proton donors, and the ether sites in the non-ionic surfactant Pluronic F127 form the proton acceptors.(Gates, Grad et al. 1994) When the CAPP system is exposed to physiological conditions, deprotonation occurs and leads to dissolution of the CAPP into its CAP and Pluronic components. This type of mechanism results in erosion-based, sustained release of drugs.(Gates, Grad et al. 1994, Raiche and Puleo 2003, Jeon, Thomas et al. 2007) CAP is commonly used as an enteric coating on tablets(Roxin, Karlsson et al. 1998), and Pluronic, an amphiphilic triblock copolymer, has been widely used for drug delivery purposes and as a surfactant. (Kabanov, Batrakova et al. 2002) *In vivo* studies have been performed with the CAPP association polymer system without any adverse effects.(Jeon, Piepgrass et al. 2008)

The CAPP polymer system has been used in the form of microspheres(Jeon, Thomas et al. 2007) and single-layered films(Xu and Lee 1993) for zero-order release of different drugs, but the present research focused on its use in multilayered devices for delivery more than one type of drug. CAPP in the form of films is more appropriate for this application, as it suits the design of multilayered devices for sequential delivery of multiple drugs. The fabrication of the CAPP films involves a relatively simple solvent evaporation technique compared with other fabrication processes, such as melt processing and injection molding, needed for other surface-eroding polymers. Fabrication of the multilayered devices involved adhering individual CAPP films using acetone as a solvent for plasticizing the surface of the CAPP films without altering the bulk properties of the film. This simple technique without the use of potentially harsh processing conditions, such high temperature and pressure, reduces the chances of small molecule drugs losing bioactivity during the fabrication process. Limitations of these CAPP films include the non-uniform distribution of hydrophilic molecules, such as proteins and lack of mechanical

flexibility of the films which is being addressed by inclusion of a plasticizing agent during the fabrication process.

3.4.1 Morphological characterization

SEM and fluorescence microscopy were used to characterize the morphological properties of the multilayered CAPP device. SEM images of the cross-section of a four-layered device showed demarcation between the CAPP layers, indicating the separation between the drug-loaded and blank layers. This type of separation between the layers was necessary to maintain the layer-based design of the device and enable sequential drug release. Cross-sectional images also showed that the small volume of solvent applied to bond layers dissolved only the surface of the films and did not affect the internal portions of the CAPP films.

Further characterization using fluorescence imaging showed clear distinction between the fluorescent and blank layers. Both qualitatively and quantitatively, fluorescence was not observed in the blank layers, which confirmed that there was no diffusion of fluorescein from loaded to unloaded layers during fabrication. An absence of interlayer diffusion even during incubation ("aging") at 37°C for six days was confirmed by the distinct separation of fluorescein-loaded and blank layers

3.4.2 Single layer drug release profiles

As indicated previously, the primary aim of this research was to fabricate a drug delivery device that serves as a platform for delivery of wide spectrum of drugs in a specified sequential order. For this purpose, some of the most commonly used drug types, such as antibiotics (metronidazole and doxycycline), anti-inflammatory agents (ketoprofen), and a potentially osteogenic small molecule drug (simvastatin), along with a model protein (lysozyme) were chosen, and their loading and release were studied. All the CAPP films with drugs were inserted into a polystyrene well to aid unidirectional polymer erosion. Results for the drugs investigated in this study showcase the ability of the CAPP system to serve as delivery platform for a variety of biomolecules.

Except for lysozyme, the other four drugs that were loaded and released are currently used for treatment in patients. Metronidazole is effective against most Gram-negative and Gram-positive anaerobic bacteria and a wide variety of protozoans.(Freeman, Klutman et al. 1997) Doxycycline is one of the commonly prescribed tetracycline

antibiotics that are effective against variety of infectious agents.(Cunha, Domenico et al. 2000) In addition, it also possesses bone anti-resorptive properties.(Vernillo and Rifkin 1998) Ketoprofen, which is commonly used for treatment of arthritis(Veys 1991) and in dentistry,(Johnny G 1988) is a phenylproprionic acid derivative with analgesic, anti-inflammatory, and antipyretic properties. The fourth small molecule drug used in this study was simvastatin, which is widely used for controlling high cholesterol level.(Todd and Goa 1990) Importantly, however, simvastatin has the ability to stimulate bone formation via enhanced expression of bone morphogenetic protein 2 (BMP-2).(Chen, Sun et al. 2010)

All four drugs were released in a sustained manner from the CAPP films and followed zero-order release kinetics. *In vitro* release results for the single layer CAPP films were statistically comparable to predictions from Hopfenberg's model developed to predict drug release from a slab.(Hopfenberg H 1976) Irrespective of the type of drug or the amount of drug that was loaded, similar release rates were measured, and nearly all of the drug was accounted for during the experiments. As a nonionic surfactant, the Pluronic F-127 component of this association polymer increases the solubilizing power of the system.(Xu and Lee 1993) This property allows a wide range of dosages to be achieved.

The model protein that was loaded into and released from CAPP films was lysozyme. Being hydrophilic, lysozyme did not dissolve completely in acetone during the fabrication process. To determine the effect of the non-uniformly distributed lysozyme particles on the release profiles, lysozyme-loaded CAPP films were eroded in two different orientations. When the film surface that was in contact with the Teflon dish (bottom surface, where the undissolved lysozyme settled during fabrication) was attached face down in the impermeable well, the top surface (with fewer lysozyme particles) eroded initially, and protein was predominantly released during the final stages of the film erosion. When the film surface that was exposed to the atmosphere (top surface) during film fabrication faced the polystyrene, release of lysozyme occurred during the initial stages of films erosion. These release profiles further confirm that release of lysozyme occurred by surface erosion of polymer, because lysozyme was released only when the part of the polymer film with high concentration of lysozyme was exposed.

3.4.3 Intermittent and sequential release of drugs

Erosion of multilayered devices *in vitro* resulted in successful release of drugs in both intermittent and sequential manners. These multilayered devices also had a polystyrene backing layer for unidirectional polymer erosion and drug release. Further research is being conducted to replace the non-degradable polystyrene with a biodegradable backing material suitable for *in vivo* implantation.

Intermittent release of the antibiotic metronidazole was achieved using an intermediate blank CAPP layer. Similarly, sequential release of an antibiotic, metronidazole, and an anti-inflammatory agent, ketoprofen, was also demonstrated by placing a blank CAPP layer between drug-loaded layers. When incubated in a smaller volume of PBS, the same devices with metronidazole and ketoprofen eroded at a slower rate. These findings show the effect that the sink has on device erosion and the consequent release profile. In both release studies, the interval at which the samples were collected was the same (every 8-10 hours). The release byproducts generated during erosion of CAPP might have saturated the smaller volume of release medium and thereby prevented (slowed) further polymer erosion. When a larger volume of release medium was used, saturation with erosion byproducts would have occurred relatively slower, thereby resulting in faster erosion. In spite of the change in erosion rate, sequential release of metronidazole followed by the release of ketoprofen was not altered. In the multilayered devices, release of the second drug occurred in a relatively more sustained manner when compared to the first drug. When multilayered devices were eroded in 4 mL of PBS, the first drug was released within 20 hours, and the second drug was released over the last 50 hours. This can be explained by the cylindrical polystyrene wells in which the layers were inserted. As CAPP eroded, PBS was retained within the well, and the reduced circulation of medium near the final layer resulted in relatively slower erosion and drug release. With only 2 mL of PBS, the first drug was released within 40 hours, and the second drug was released over the last 100 hours. In this case, the combined effects of the reduced sink conditions and reduced mixing within the wells further slowed erosion, but the total amount of drug released was not significantly affected. Mass balance calculations again showed comparable expected and observed drug amounts loaded and released. Hopfenberg's equation was also suitable for modeling sequential release of more than one drug from the

multilayer CAPP device. The predicted profiles also showed that the rate of release of the first drug was approximately twice as fast as that for the second drug. The predicted and experimentally measured release profiles were statistically similar for both the single layer and multilayered devices, indicating the suitability of this particular model for the present delivery system. This model for predicting the release profiles from CAPP devices would be helpful for further design of advanced multilayered devices.

Mass loss profiles showed that CAPP devices, irrespective of the type of drugs loaded, followed a surface-erosion pattern. As such, the duration of the drug release as well as the time interval between the release peaks of the same or different drugs can be increased or decreased by altering the thickness of the blank CAPP films. Overall, the type and amount of drug loaded in the CAPP films can be altered to achieve a desired intermittent or sequential release using this multilayered system.

3.4.4 Bioactivity of released protein

Because proteins are more unstable compared to small molecule drugs, initial bioactivity testing was conducted for only the protein that was released from the CAPP films. Results showed that lysozyme lost approximately 40% of its activity during loading and delivery. Other commonly used bioerodible/biodegradable delivery systems, such as poly(lactic-co-glycolic acid) (PLGA) and poly(ethylene gycol) (PEG), also result in loss of protein activity by aggregation, hydrolytic degradation, and chemical modification during the necessary manufacturing process, which can involve heating, pH changes, shear forces, organic solvents, drying and others.(Gombotz and Pettit 1995) With the CAPP delivery system, however, the amount of protein loaded can be easily altered, and excipients may enhance preservation of bioactivity.

The present studies have shown that CAPP film-based devices can be used to deliver a wide variety of drugs and can be used to achieve sequential delivery of multiple drugs. These advantages will be helpful for customizing devices different applications. For example, some bacterial infections, which might involve more than one type of microorganism, require combination of antibiotics for treatment(Griffiths, Ayob et al.) to eliminate the infection and reduce the potential for developing antibiotic resistance.(Dowling 1957) The ability of this CAPP system to sequentially deliver anti-inflammatory agents with other drugs creates the possibility of inflammation control and

pain management during wound healing. The system may also be useful for delivery of multiple growth factors for tissue engineering. A growing body of research suggests the importance of more than one growth factor for regeneration of tissues, such as bone,(Jeon and Puleo 2008) (Raschke, Wildemann et al. 2002) (Yilgor, Tuzlakoglu et al. 2009) blood vessels,(Richardson, Peters et al. 2001) and cartilage.(Elisseeff, McIntosh et al. 2001) (Fukumoto, Sperling et al. 2003)

There have been several attempts towards the delivery of multiple growth factors.(Chen, Chen et al.) Most of these approaches, however, have been successful for simultaneous delivery.(Lynch, de Castilla et al. 1991, Schmidmaier, Wildemann et al. 2002, Nevins, Camelo et al. 2003, Simmons, Alsberg et al. 2004, Dogan, Gumusderelioglu et al. 2005, Peattie, Rieke et al. 2006, Riley, Fuegy et al. 2006, Nillesen, Geutjes et al. 2007, Patel, Young et al. 2008, Chen, Chen et al. 2009, Young, Patel et al. 2009, Borselli, Storrie et al.) Temporally controlled release has been obtained by other strategies, including fabrication scaffolds and/or microspheres consisting of one or more polymers each loaded with a different drug, *e.g.*, PLGA in combination with gelatin hydrogels, poly(propylene fumarate), poly(4-vinyl pyridine), alginic acid, cellulose acetate.(Chen, Silva et al. 2007, Buket Basmanav, Kose et al. 2008, Jaklenec, Hinckfuss et al. 2008, Kempen, Lu et al. 2009, Tengood, Kovach et al. 2010) The system presented in this paper was composed of a single association polymer system in the form of multilayered films, which simplifies the fabrication process and eases loading of wide variety of drugs to obtain localized delivery of multiple drugs in a required temporal sequence.

3.5 Conclusion

The easy to fabricate CAPP association polymer can be used to achieve zero-order release of a wide variety of drugs. As such, this system can serve as a general platform for localized, controlled drug delivery to treat several disease conditions. Different release profiles can be designed, including sustained release of one drug, intermittent release of a drug, or sequential release of multiple drugs. This system could be used for applications that require delivery of more than one type drug in a predetermined temporal sequence.

Chapter 4 Design of a Multiple Drug Delivery System Directed at Periodontitis

4.1 Introduction

Periodontitis is one of the most common inflammatory diseases and is a leading cause of tooth loss in adults (Brown, Oliver et al. 1989, Albandar and Kingman 1999). It is also related to systemic disorders, such as coronary artery disease, stroke, and diabetes (Soskolne and Klinger 2001, Chen, Chen et al. 2009). In the initial stages of periodontitis, the onset of bacterial infection is followed by the host response of active and progressive inflammation, leading to resorption and loss of tissue (Dionne and Berthold 2001). When periodontitis is well-established, effective therapeutic and surgical intervention is required for the removal of bacterial plaque, control of inflammation, and inhibition of progressive bone loss with subsequent complete repair and regeneration of functional periodontium (Chen, Chen et al. 2009). One of the most common methods for treating chronic periodontitis involves mechanical debridement of periodontal pockets by scaling and root planning along with effective plaque control to eliminate bacterial infection (Etienne 2003). Subsequent periodontal regenerative procedures are time-consuming and financially demanding (Polimeni, Xiropaidis et al. 2006), and currently there is no ideal therapeutic approach to completely cure periodontitis and achieve predictable tissue regeneration (Chen, Chen et al. 2009). Because the progression of periodontitis involves a complex, sequential relationship between infection, inflammation, and tissue loss (Caton, Ciancio et al. 2000), treatment might be improved by controlled release of multiple biologically active agents in an appropriate sequence (Cochran D L. (2003)).

Vyas et al. reviewed controlled drug delivery systems that have been employed for treating periodontal diseases (Vyas, Sihorkar et al. 2000). Some approaches involved localized delivery of antibiotics for elimination of bacterial infection (Schwach-Abdellaoui, Vivien-Castioni et al. 2000, Etienne 2003), while others have addressed inflammation (Queiroz-Junior, Pacheco et al. 2009, Srinivas, Medaiah et al. 2011) or bone resorption (Binderman, Adut et al. 2000). Periodontal regeneration has also been attempted using local delivery of osteogenic agents (King, King et al. 1998, Selvig, Sorensen et al. 2002, Yazawa, Zimmermann et al. 2005, Seto, Ohba et al. 2008). None of these methods, however, addressed all aspects of the disease to achieve comprehensive treatment.

The present research was aimed at developing an “all-encompassing”, multiple drug delivery system capable of delivering antibacterial, anti-inflammatory, anti-resorptive, and osteogenic agents in the appropriate sequence for potential treatment of periodontitis. Figure 4.1 shows a schematic representation of the order in which the drugs will be delivered at different stages based on pathogenesis of the disease.

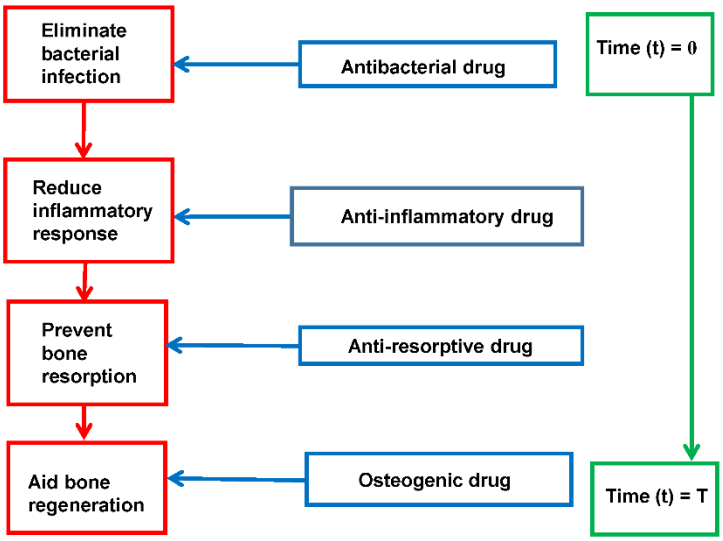


Figure 4.1: Proposed sequential drug delivery based on the pathogenesis of periodontal disease.

4.2 Materials & Methods

4.2.1 Fabrication of multilayered devices

Devices were fabricated using a surface-eroding association polymer system (CAPP) comprising cellulose acetate phthalate (CAP) (Sigma-Aldrich, St. Louis, MO) and Pluronic F-127 (P) (Sigma-Aldrich) (Xu and Lee 1993, Raiche and Puleo 2003). CAPP films, prepared by a solvent evaporation technique, were used to fabricate the multilayer devices. CAP and Pluronic F-127 were mixed together in the weight ratio of 70:30, respectively, and dissolved in acetone to obtain an 8% polymer solution. The drug of interest (5 wt %) was added to the acetone-polymer solution and mixed thoroughly until the drug was completely dissolved. The drug-polymer solution was poured in a Teflon dish and stored at 4ºC for 24 hours for slow evaporation of the solvent. Blank CAPP films were prepared in the same way but without the addition of drugs. For this study, CAPP films were loaded with metronidazole (Sigma-Aldrich), ketoprofen (Sigma-Aldrich), doxycycline (Sigma-Aldrich), or simvastatin (Haorui Pharma-Chem, Inc., Edison, NJ). Samples with diameter of around 6 mm and thickness of 0.5 mm were punched out of the CAPP films. The drug-loaded discs were arranged in the desired sequence with alternating layers of blank CAPP films (Figure 4.2).

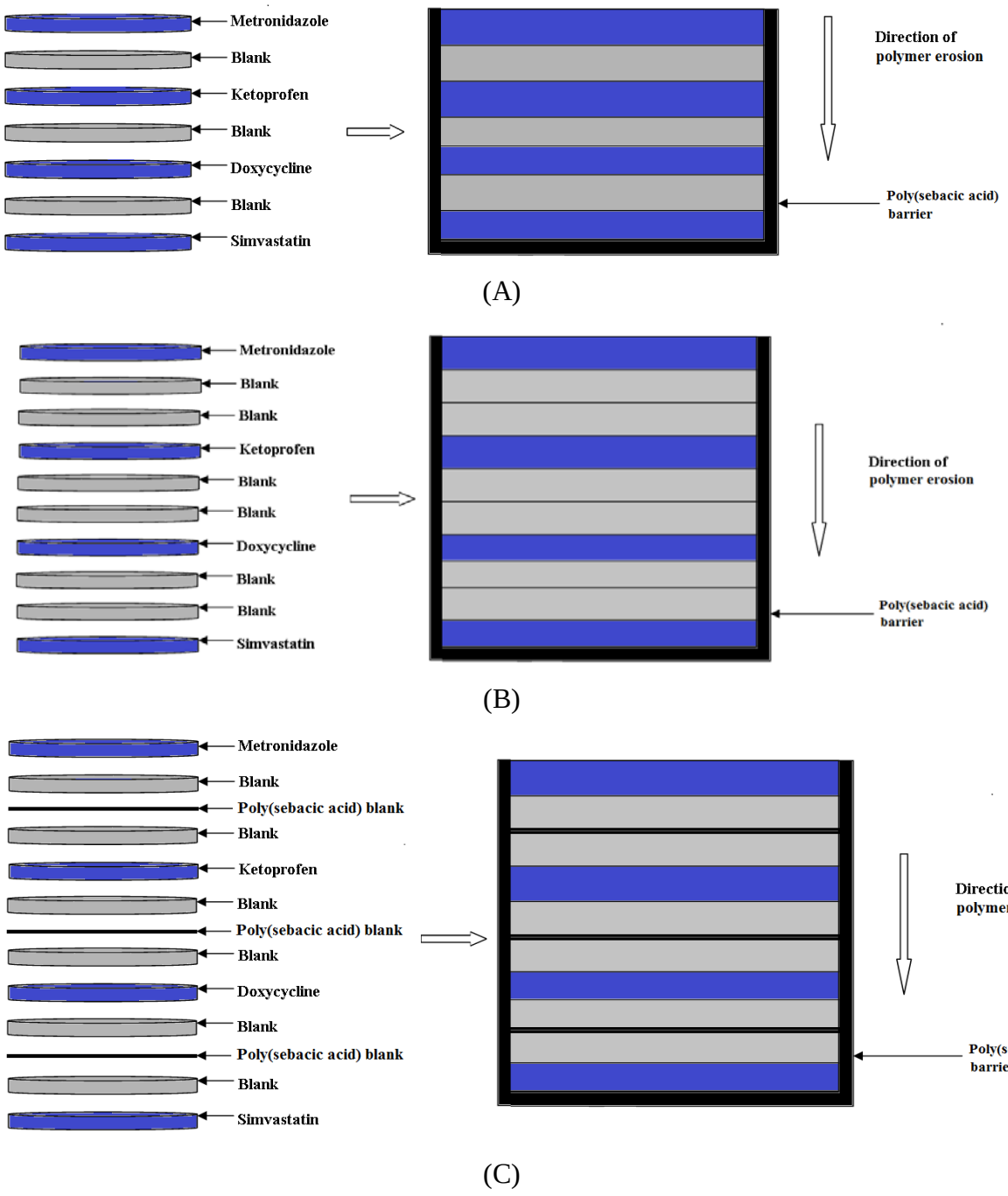


Figure 4.2: Schematic representation of how multilayered CAPP devices were fabricated. (A) 7-layer device with one blank layer between drug layers. (B) 10-layer device with two blank layers between drug layers. (C) 10-layer device with PSA layer between the blank layers. Note: Illustration is not to scale.

The stack of the CAPP films was bonded together by compressing them after acetone had been applied between the layers. The multilayered device was then coated with poly(sebacic acid) (diacetoxy-terminated; PSA; Sigma-Aldrich), which acted as a barrier to enable unidirectional erosion and drug release. Blank (drug-free) multilayered devices were used for comparison. Three different device designs were investigated for increasing the duration of erosion and release. In addition to the single CAPP blank layers, either two blank layers were used or a thin PSA layer was included between the blank layers.

4.2.2 Mass loss and drug release

The multilayered devices were eroded in phosphate-buffered saline (PBS), pH 7.4, during incubation at 37ºC with gentle shaking. After collecting the supernatants at regular time intervals, the samples were weighed and then fresh PBS was added. The measured mass of the samples was used to construct the mass loss profiles of the multilayered CAPP devices. Collected supernatants were used to determine the amount of metronidazole, ketoprofen, doxycycline, and simvastatin using high performance liquid chromatography (HPLC; Shimadzu Prominence). For measuring the concentration of ketoprofen, an isocratic mobile phase composed of acetonitrile (60%) and 0.1% trifluroacetic acid (TFA) in DI (deionized) water (40%) was used with UV detection at 260 nm, and for simvastatin, the isocratic mobile phase was acetonitrile (70%) and 0.1% TFA in DI water (30%) with UV detection at 240 nm. A gradient mobile phase with acetonitrile and 0.1% TFA in DI water was developed for measuring the concentration of metronidazole and doxycycline with UV detection at 318 and 350 nm, respectively.

4.2.3 Mathematical modeling

Profiles of drugs released from the multilayered CAPP device weres evaluated using Hopfenberg's model for controlled release from erodible slabs (Eq. 1):

$$\frac{M_t}{M_\infty} = 1 - \left[1 - \frac{k_0\, t}{C_0\, a}\right] \qquad (1)$$

where M_t is the amount of drug released (mg) at time t (hours), M_∞ the total amount of drug released from the device (mg), k_o the erosion constant (mg/hr/mm^2), C_o initial concentration of the drug in the device (mg/mm^3), a the half thickness of the slab, and n=1 for a slab (Hopfenberg H 1976). Because only one side of the CAPP layer (slab) was exposed for polymer erosion and drug release due to the presence of the PSA barrier, the

term a (half the thickness of the slab) was replaced with 2a (total thickness of the slab in mm) in equation (1). The release profiles predicted using this mathematical model were compared with the experimentally-obtained cumulative release profiles of the four drugs.

4.2.4 Bioactivity

Bioactivity of released metronidazole or ketoprofen was measured to assess effects of encapsulation and release. The Kirby-Bauer assay was performed to test the antibacterial activity of metronidazole. An aliquot of *Porphyromonas gingivalis* (FDC381) (*P. gingivalis*) culture was uniformly spread on blood agar plates using polystyrene beads. Release supernatant (7 µL) containing metronidazole was added to 7 mm diameter filter paper discs and placed on the *P. gingivalis*-inoculated plates. After 24 hour incubation under anaerobic conditions, the plates were imaged, and the area of inhibition (clear zone) around the filter papers was measured using ImageJ software. Results from the release supernatants were compared to the clear zones obtained using serial dilutions of fresh antibiotic to determine the percent bioactivity. A cyclooxygenase (COX) inhibitor assay kit (Cayman Chemical Company, Ann Arbor, MI) was used to determine the bioactivity of the ketoprofen released from the CAPP films. Activity against COX-1 enzyme was measured using the manufacturer's protocol. As for metronidazole, COX inhibition by the release supernatants was compared with that of freshly prepared standard dilutions of ketoprofen to determine the percent bioactivity.

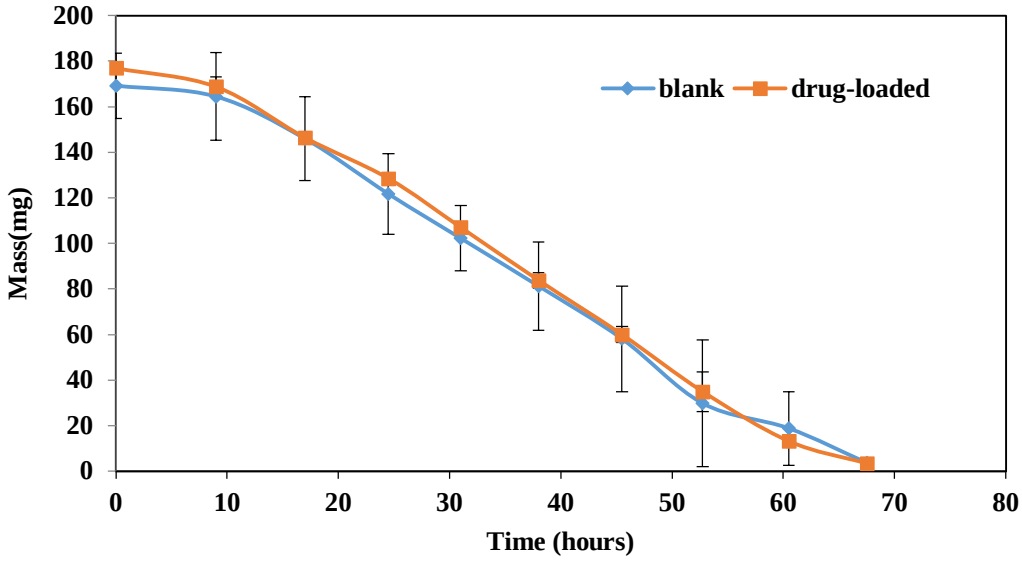
4.2.5 Statistical analysis

Mass loss and drug release profiles, both experimental and predicted from mathematical modeling, were analyzed by linear regression using GraphPad Prism software (La Jolla, CA). Statistically significant differences between the bioactivity of the "fresh" drugs and the those released from the CAPP films were determined using the Student's t-test (InStat, GraphPad Software).

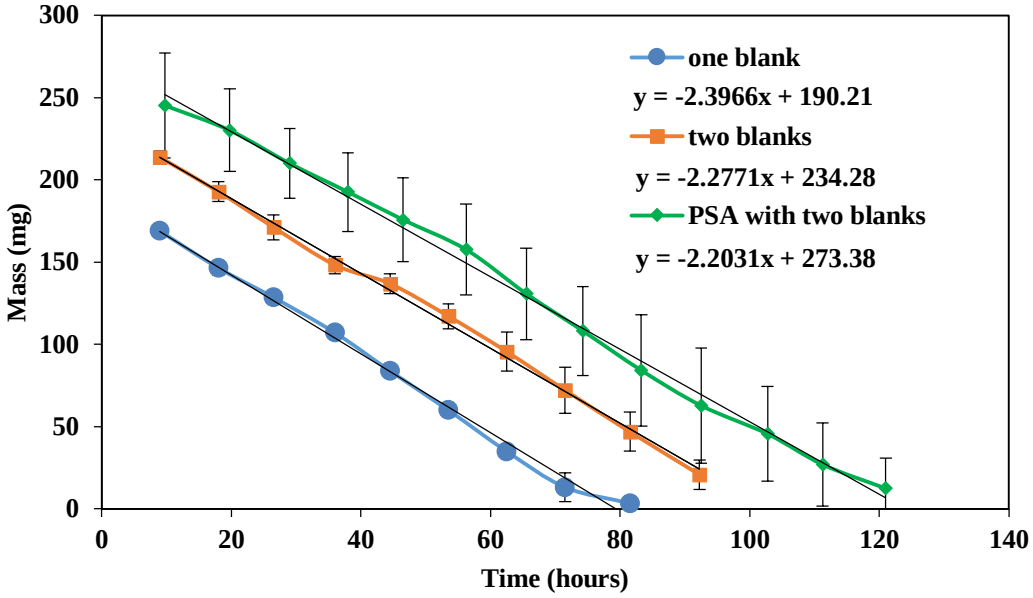
4.3 Results

4.3.1 Mass loss profiles

Figure 4.3 shows the mass loss profiles for the multilayered CAPP devices when eroded in PBS. Both the control (blank) devices without drug and the drug-loaded CAPP devices followed similar linear erosion profiles (Figure 4.3 A). There was no statistically significant difference between the slopes of the mass loss curves during the course of the study. Mass loss profiles of the multilayered devices with single blank layers, two blank layers, and with PSA blank layers are shown in Figure 4.3 B. Comparison of the slopes of the mass loss curves also showed that there were no statistically significant differences in the rate of erosion between the different types of devices. The y-intercept of the lines indicates the average initial mass of the different devices. On average, mass loss occurred at a rate of 0.08 mg/mm^2/hour when sink conditions were maintained by replacing PBS at regular time intervals.



(A)



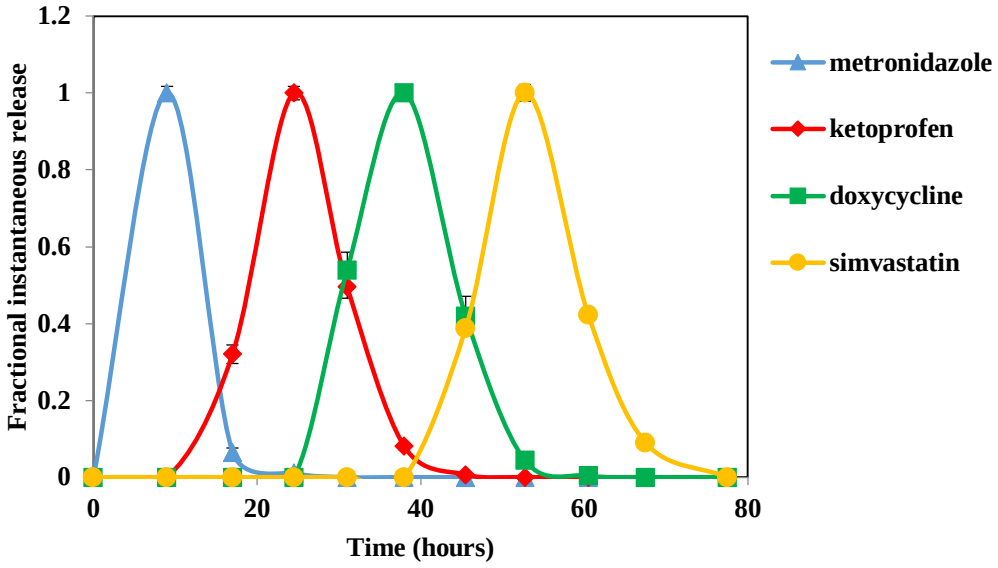
(B)

Figure 4.3: Mass loss profiles for: (A) 7-layered blank and drug-loaded CAPP devices; (B) Drug-loaded devices with one blank layer, two blank layers, or two blank layers along with PSA between the drug layers. Data are mean ± standard deviation (n=3).

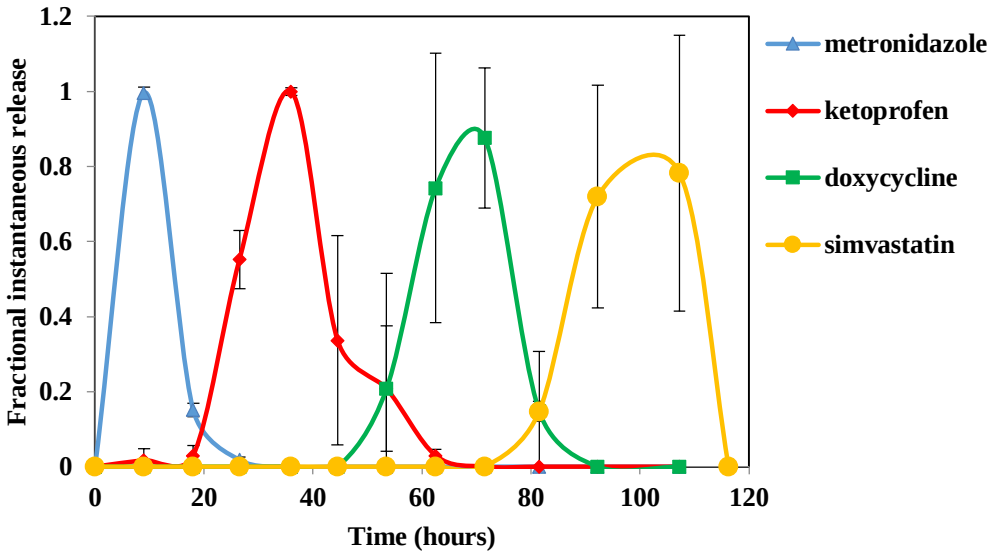
4.3.2 Drug release profiles

Figure 4.4 A shows the instantaneous profiles for four drugs released from the multilayered delivery system. Because metronidazole was loaded in the layer initially exposed when erosion started, it was the first drug released within 15-20 hours of incubation in PBS. This was followed by release of the anti-inflammatory drug ketoprofen during the course of further device erosion. Ketoprofen release finished midway (around 40 hours) through the total device erosion time (70-80 hours). The third drug released from the system was doxycycline, which started around 25 hour and lasted through 50 hours. The last drug, simvastatin, was released during the final stages of device erosion (50-80 hours).

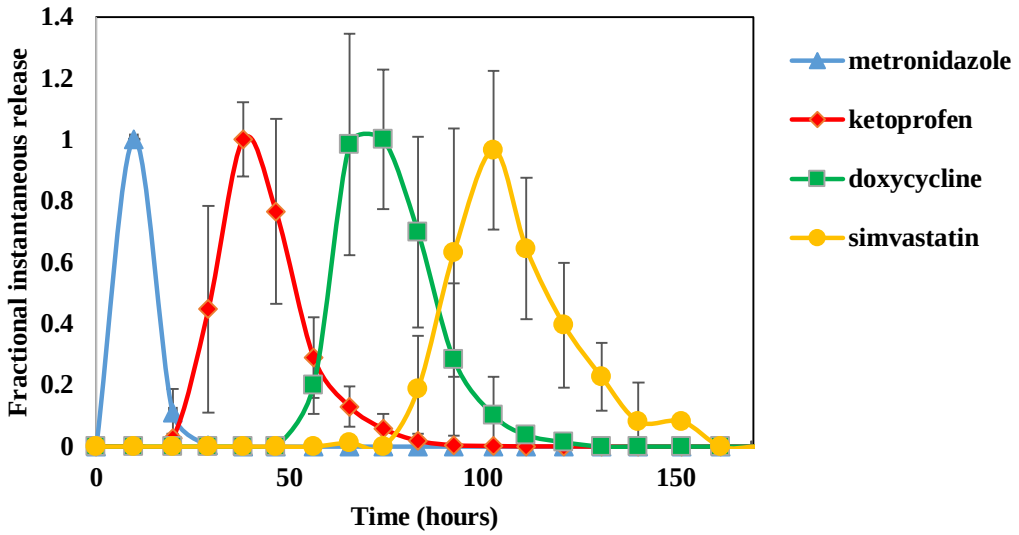
The multilayered devices fabricated with two blank layers, instead of just one, between the drug-loaded layers showed similar sequential release of four drugs (Figure 4.4 B). With two blank layers, however, the separation between the drug release peaks was more distinct, and the erosion time of the whole device was increased to 120 hours. In this case, the first drug, metronidazole, was released within the first 25 hours followed by the release of ketoprofen through 60 hours, which was half the erosion time of the device. Release of the third drug, doxycycline, started at around 40 hours and lasted until 100 hours, and this was followed by release of simvastatin through complete erosion of the devices at 120 hours.


Fractional instantaneous release
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
Time (hours)
metronidazole
ketoprofen
doxycycline
simvastatin

(A)


Fractional instantaneous release
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
Time (hours)
metronidazole
ketoprofen
doxycycline
simvastatin

(B)



(C)

Figure 4.4: Fractional instantaneous release profiles of four drugs from: (A) 7-layer devices with single blank layers; (B) 10-layer devices with two blank layers; and (C) 10-layer devices with double blank layers plus PSA. Data are mean ± standard deviation (n=3).

The use of the PSA layers between the CAPP blank layers further increased the erosion time of the device to 160 hours. Release of metronidazole occurred within the first 25 hours, as in the case of the other two device types. But the presence of PSA layers before the other drug layers slowed erosion and extended the release periods of ketoprofen, doxycycline, and simvastatin to 20-80, 50-100, and 90-160 hours, respectively. The times at which the peaks were observed for all four drugs in the three different device types are given in Table 4.1.

Table 4.1: Time of metronidazole, ketoprofen, doxycycline, and simvastatin peaks for the three types of devices fabricated and tested.

Device type	Metronidazole (hour)	Ketoprofen (hour)	Doxycycline (hour)	Simvastatin (hour)	Total erosion time (hours)
One blank	9	24	38	53	78
Two blanks	9	36	70	100	116
PSA with two blanks	10	38	74	103	161

4.3.3 Mathematical modeling and mass balance

In vitro release of four drugs from the multilayered devices was compared to the profiles predicted using Hopfenberg's model. Figure 4.5 shows representative results for the double blank layer devices. The predicted release profiles were similar to those measured for sequential release of the four drugs from the multilayered CAPP devices. Linear regression showed that there were no statistically significant differences between the slopes of the predicted and experimental drug release profiles. The measured amounts of drugs released were not significantly different from the expected amounts.

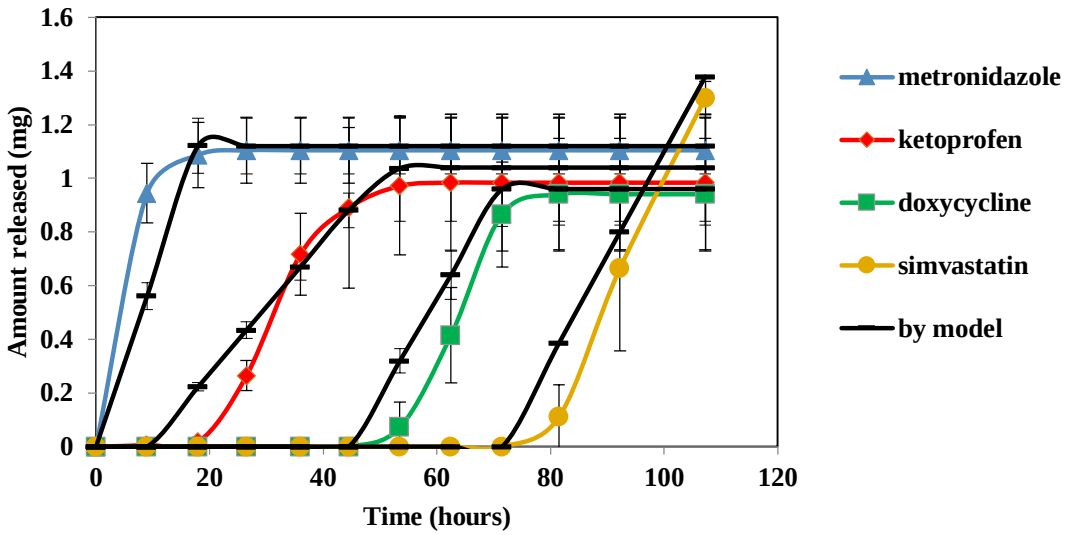


Figure 4.5: Cumulative drug release from double blank layer devices along with mathematical modeling.

4.3.4 Bioactivity

Figure 4.6 shows the bioactivity of the metronidazole supernatants at 1, 3, and 5 hours of release from single-layered CAPP films as measured by the ability to kill *P. gingivalis*. The results of the Kirby-Bauer assay showed that the observed area of inhibition caused by the metronidazole supernatants from all the release/erosion supernatants was about 10% higher than that expected from fresh antibiotic solutions of the same concentration. Even though the observed area of inhibition was higher than expected, statistical analysis showed that the difference was statistically significant at only the 5 hour time point ($p = 0.0152$). Release supernatants from blank CAPP films, which were used as controls, did not produce any clear area (not shown).

By measuring inhibition of COX-1 enzyme activity, ketoprofen in release supernatants was also found to have retained its bioactivity (Figure 6). Even though the assay results showed that the ketoprofen from the release supernatant had more than 100% bioactivity retention, about 120% on an average, statistical analysis showed that there were no significant differences.

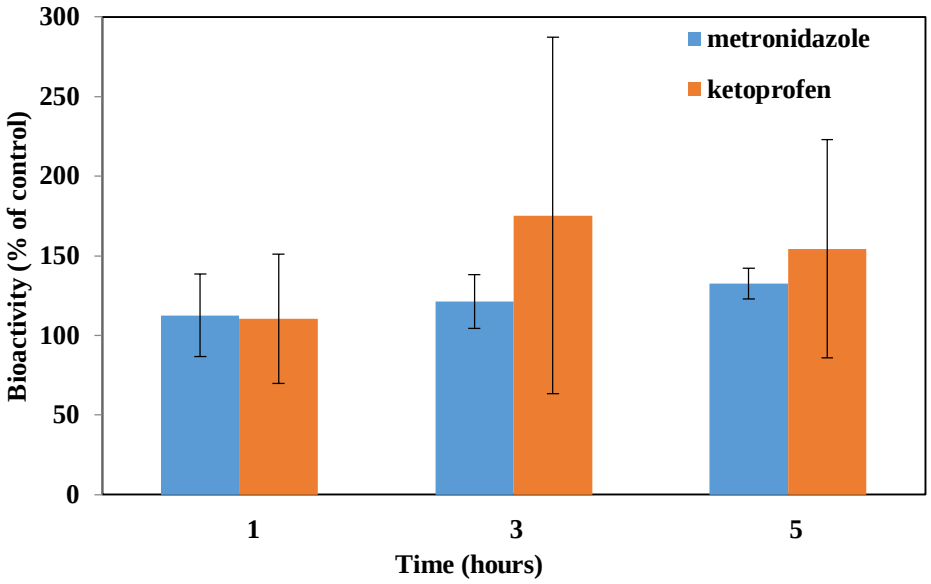


Figure 4.6: Percentage of bioactivity retained by metronidazole and ketoprofen released from CAPP films. Data are mean ± standard deviation (n=3).

4.4 Discussion

The complexity of periodontal pathogenesis necessitates use of more than one type of drug for complete treatment of the condition. For this purpose, a localized delivery system capable of releasing multiple drugs in a sequential order would be ideal. Chen et al. (Chen and Jin 2010) and Santo et al. (Santo, Gomes et al. 2013) have given a detailed account of current research on multiple drug delivery systems for tissue engineering. The majority of these multiple drug delivery systems were designed for release of more than one type of drug simultaneously (Lynch, de Castilla et al. 1991, Simmons, Alsberg et al. 2004, Dogan, Gumusderelioglu et al. 2005, Peattie, Rieke et al. 2006, Patel, Young et al. 2008, Chen, Chen et al. 2009, Borselli, Storrie et al. 2010) or sequential release of more than one type of drug by using composite devices comprising more than one polymer (Chen, Silva et al. 2007, Buket Basmanav, Kose et al. 2008, Jaklenec, Hinckfuss et al. 2008, Kempen, Lu et al. 2009, Tengood, Kovach et al. 2010). The multilayered delivery devices designed for the present research using a single association polymer system successfully released four different drugs in the desired temporal sequence for potential treatment of periodontitis. The sequence in which these four drugs are delivered is of critical importance for the treatment of the disease. For example, administration of an osteogenic agent before elimination of the bacterial infection and inflammation would not be as effective for tissue regeneration.

Delivery devices based on the CAPP association polymer system have been used for drug release both in the form of films and microspheres (Gates, Grad et al. 1994, Jeon, Piepgrass et al. 2008). For the present research, the physical form of films was selected for designing a system for sequential drug release. Previous research demonstrated that CAPP films can deliver drugs in a near-zero-order fashion(Sharath C. Sundararaj 2013). In agreement, the mass loss profile followed a linear pattern with the mass loss occurring at a rate of 2.5 mg/hour, suggesting surface erosion and drug release. Use of the surface-eroding polyanhydride poly(sebacic acid), which erodes more slowly than does CAPP, as a barrier layer limited erosion to only one surface and, therefore, unidirectional erosion and drug release.

Selection of the appropriate drugs is also an important factor for successful therapy. The first step in the treatment of periodontitis involves elimination of the bacterial

infection. The initial stages of wound management are critical for repair and regeneration of periodontal tissues, because the innate regenerative potential of the periodontium is dependent on wound stability (Polimeni, Xiropaidis et al. 2006). The localized delivery of various antimicrobial agents for treatment of chronic periodontitis and its advantages have been discussed by Etienne (Etienne 2003). Of the various antimicrobial agents used, including tetracycline, chlorhexidine, doxycycline, and minocycline (Etienne 2003), metronidazole was chosen for this research because it is one of the most commonly used antibiotics against periodontal pathogens. Metronidazole concentrations achieved by systemic administration might not be effective against biofilm-associated *P. gingivalis* (Wright, Ellen et al. 1997), however, which further increases the need for localized antibiotic delivery for the treatment of chronic periodontal conditions. *Porphyromonas gingivalis* is a pathogenic bacterium commonly associated with periodontal infections (Tribble, Lamont et al. 2007, Japoni, Vasin et al. 2011). Metronidazole has been shown to significantly reduce *P. gingivalis* infection compared to other antibiotics *in vitro* (Eick and Pfister 2004), and it is effective against different strains (Larsen 2002). Localized delivery of metronidazole has been achieved in the form of gels for treatment (Sato, Fonseca et al. 2008), and studies have shown that metronidazole can readily attain minimum inhibitory concentrations in gingival tissue and crevicular fluid (Van Oosten, Notten et al. 1986, Tenenbaum, Cuisinier et al. 1993). Besides gels, metronidazole has also been loaded and released using CAPP films by Gates et al. (Gates, Grad et al. 1994) and by the authors (Sharath C. Sundararaj 2013). The effectiveness of metronidazole as an antimicrobial agent against periodontal pathogens and its successful localized delivery using CAPP films makes it an appropriate option for the first drug to be delivered for the treatment of periodontitis.

The bacterial infection associated with periodontitis results in an exuberant host response, with chronic inflammation leading to the loss of soft tissue and resorption of the alveolar bone (Williams 1990). The persistence of inflammation will affect the regenerative process even if growth factors or other osteogenic agents are provided (Caton, Ciancio et al. 2000). Consequently, the inflammatory response must be controlled before attempting to prevent bone resorption and aid regeneration. The present CAPP system was capable of releasing the anti-inflammatory drug ketoprofen before delivering antiresorptive

and osteogenic agents. Ketoprofen has been successfully used in the treatment of periodontitis (Paquette, Fiorellini et al. 1997, Salvi and Lang 2005). Both systemic and topical administration of ketoprofen in the form of gels was effective in reducing prostaglandin levels in gingival crevicular fluid in adult periodontal patients, which thus aided inhibition of disease progression (Lawrence, Paquette et al. 1998, Paquette, Lawrence et al. 2000). Both metronidazole and ketoprofen retained essentially 100% of their bioactivity, which shows that the loading of drugs in the CAPP films and their subsequent release did not adversely affect the drugs.

After ketoprofen, doxycycline was released from the CAPP delivery system. Doxycycline, a tetracycline derivative, has significant anti-matrix metalloproteinase activity and also inhibits osteoclast development, structure, and function (Vernillo and Rifkin 1998). Furthermore, doxycycline also has antimicrobial properties and has been shown to be effective against *P. gingivalis* (Larsen 2002). Llindhe et al. showed the effectiveness of locally delivered tetracycline using hollow fibers in periodontal pockets for the elimination or reduction of clinical symptoms of periodontitis (Llindhe, Heijl et al. 1979). These factors make doxycycline an appropriate third drug released to inhibit tissue loss. The antimicrobial properties of doxycycline along with metronidazole which was delivered first, provide continuous protection against bacteria at the site of repair and regeneration. In the case of severe bacterial infection, a combination of antibiotics might be more effective for periodontal treatment (Griffiths, Ayob et al. 2011), and this type of multiple antibiotic delivery can be readily achieved using this CAPP system.

Simvastatin was the final drug released from the CAPP system. Beside its common use for cholesterol control (Todd and Goa 1990), simvastatin serves as an osteogenic agent (Chen, Sun et al. 2010). Apart from aiding general bone regeneration by increasing the expression of BMP-2 (Mundy, Garrett et al. 1999), simvastatin has been specifically used for periodontal regeneration (Pradeep and Thorat 2010). Simvastatin is a cost-effective option when compared to BMP-2, and topical administration of simvastatin has the potential to effectively recover alveolar bone loss (Seto, Ohba et al. 2008). Simvastatin has been shown to have a positive effect on the periodontal ligament cells (Yazawa, Zimmermann et al. 2005), which have the capacity to regenerate the periodontal attachment (Karring, Nyman et al. 1993). Other studies have shown that simvastatin inhibits

inflammation and encourages angiogenesis, which might have further positive effects on bone formation (Edwards and Spector 2002). Simvastatin has been loaded into and released from CAPP microspheres m compressed in the form of films for *in vivo* bone regeneration (Jeon, Piepgrass et al. 2008). Along with its effectiveness in overall periodontal regeneration, prior knowledge on delivery of simvastatin using CAPP system makes it a suitable final drug to be released from this multilayered CAPP delivery system.

Two main advantages of this system include: 1) the relative ease with which the CAPP films can be fabricated using the solvent evaporation technique and 2) the capability of the devices to release a variety of bioactive drugs in a sequential manner. The dose of drugs loaded in the CAPP films can be altered based on the severity of the condition and the type of drug used. The time of drug release can also be controlled by altering the thickness of the layers of CAPP films. Use of two blank layers instead of one resulted in more distinct separation of drug release peaks and also increased the total erosion time of the devices, in spite of slight overlapping that was observed between the adjacent drug release peaks. The use of approximately 300 µm thick PSA layers between the CAPP blank layers further increased the total erosion time of the device by approximately a factor of two in comparison with the devices with single blank layers, with only a slight increase in the overall thickness. Thus, the PSA layers can be increased in thickness or they can replace the blank CAPP layers completely to achieve an even longer erosion time, depending on the treatment requirements. Adjustments in the device design make it possible to achieve different erosion times as well as different release times for the drugs. This type of versatility will be critical in designing devices based on the specificity of disease conditions.

4.5 Conclusions

The multilayered, bioerodible CAPP delivery system was successful in releasing four different drugs in a predetermined temporal sequence based on the pathogenesis of periodontitis. This device serves as an initial step in the development of multiple drug delivery systems for use against periodontitis or other complex disease condition, which ideally will intervene at different stages of the disease to provide a more comprehensive treatment for the condition.

Chapter 5 Comparison of *In vitro* and *In vivo* Sequential Drug Release

5.1 Introduction

The transition of a polymer-based drug delivery device from *in vitro* studies to *in vivo* testing is an important step in characterization of the device properties and performance. There is a vast amount of reviews available on the release of drug molecules from polymeric devices *in vitro* (Langer and Chasin 1990, Schacht 1990, Jain, Yenet Ayen et al. 2011), most of these *in vivo* studies have been logically aimed at determining effectiveness of the drug released for treating a particular condition or regeneration of a tissue, but not necessarily the *in vivo* drug release pattern. Comparatively there are few published studies available on *in vivo* biomaterial degradation/erosion and drug release profiles from these advanced devices (Schmidt, Wenz et al. 1995, Mäder, Crémmilleux et al. 1997, Avgoustakis, Beletsi et al. 2002, Yang, Chu et al. 2002, Mittal, Sahana et al. 2007, Zolnik and Burgess 2008, Evren ALĞIN YAPAR 2010, Mashayekhi, Mobedi et al. 2013).

Due to the highly dynamic physiological environment in which the device would be implanted, the material would be exposed to different cell types and numerous biomolecules (Hutmacher, Hurzeler et al. 1996), and studies have shown the effect of enzymes on biodegradable polymers, such as poly(glycolic acid) and poly(lactic acid) (Williams and Mort 1977, DF. 1981). The biodegradable polymers used for drug delivery follow different degradation rates *in vivo* when compared to *in vitro* conditions (Domb and Nudelman 1995, Hutmacher, Hurzeler et al. 1996, Tracy, Ward et al. 1999, Bolgen, Menceloglu et al. 2005, Zolnik and Burgess 2008, Lockwood, Hergenrother et al. 2010), and these differences would ultimately result in altered drug release profiles. Understanding of *in vivo* drug release will be useful for improving the device, providing data by which delivery can be tailored more specifically for its intended purpose in disease treatment or tissue regeneration.

The surface-eroding CAPP association polymer system comprising cellulose acetate phthalate (CAP) and Pluronic F-127 (P) was previously designed to deliver four different drugs in a particular sequence aimed at treating periodontitis in a step-by-step manner (Sharath C. Sundararaj 2013). In the present studies, after *in vitro* characterization of the CAPP system by *in vitro* mass loss and drug release measurements, *in vivo* erosion

and drug release properties were analyzed. The main aim of this research was to compare the *in vitro* and *in vivo* behaviors of a multilayered device with the capability of sequential release.

5.2 Materials & Methods

5.2.1 Fabrication of multilayer device

CAPP films were prepared by solvent evaporation after mixing CAP (Sigma-Aldrich, St. Louis, MO) and Pluronic F-127 (Sigma-Aldrich) together in the weight ratio of 90:10, respectively. The mixture was dissolved in acetone to obtain an 8% polymer solution followed by the addition and dissolution of the drug of interest (1 or 5 wt%) in the polymer solution. The drug-polymer solution was poured in a Teflon dish and stored at 4ºC for 24 hours for slow evaporation of the solvent. Blank CAPP films were prepared in the same way but without the addition of drugs. For the present study, CAPP films were loaded with metronidazole (Sigma-Aldrich), ketoprofen (Sigma-Aldrich), doxycycline (Sigma-Aldrich), or simvastatin (Haorui Pharma-Chem, Inc., Edison, NJ). Samples with diameter of around 6 mm and thickness of 0.5 mm were punched out of the CAPP films. The drug-loaded discs were arranged in the desired sequence with alternating layers of two blank CAPP films to fabricate multilayered devices. The stack of the CAPP films was bonded together by application of acetone between the layers. The multilayered device was then coated with poly(sebacic acid) (diacetoxy-terminated; PSA; Sigma-Aldrich), which acted as a barrier to enable unidirectional erosion and drug release. The completed device was wound with 4-0 poly(glycolic acid) (PGA) (Oasis, Mettawa, IL) suture for circumferential reinforcement followed by compression at 1,500 N using a BOSE ELF 3300 to consolidate the layers and reduce the overall thickness of the device. Blank (drug-free) multilayered devices were used for comparison. To increase the duration of erosion and release, a second type of device was fabricated with a thin PSA layer included between the two blank layers. Figure 5.1 shows a schematic representation of the process involved in fabrication of the two types of multilayered CAPP devices. For fabrication of the devices used for, the CAPP films and PSA were UV-sterilized for 1.5 hours, the solvents sterile-filtered, and assembly conducted in a laminar flow hood.

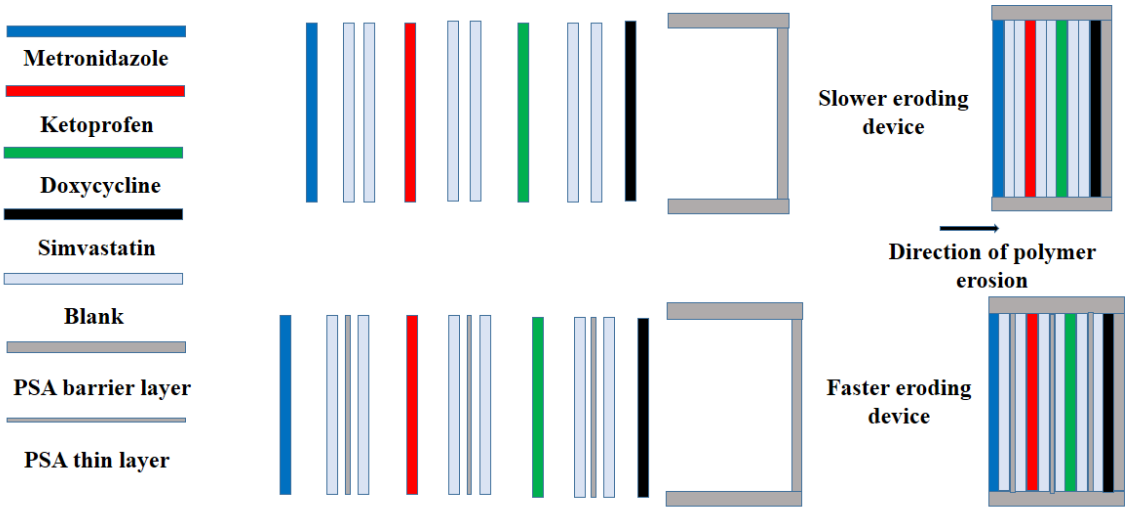


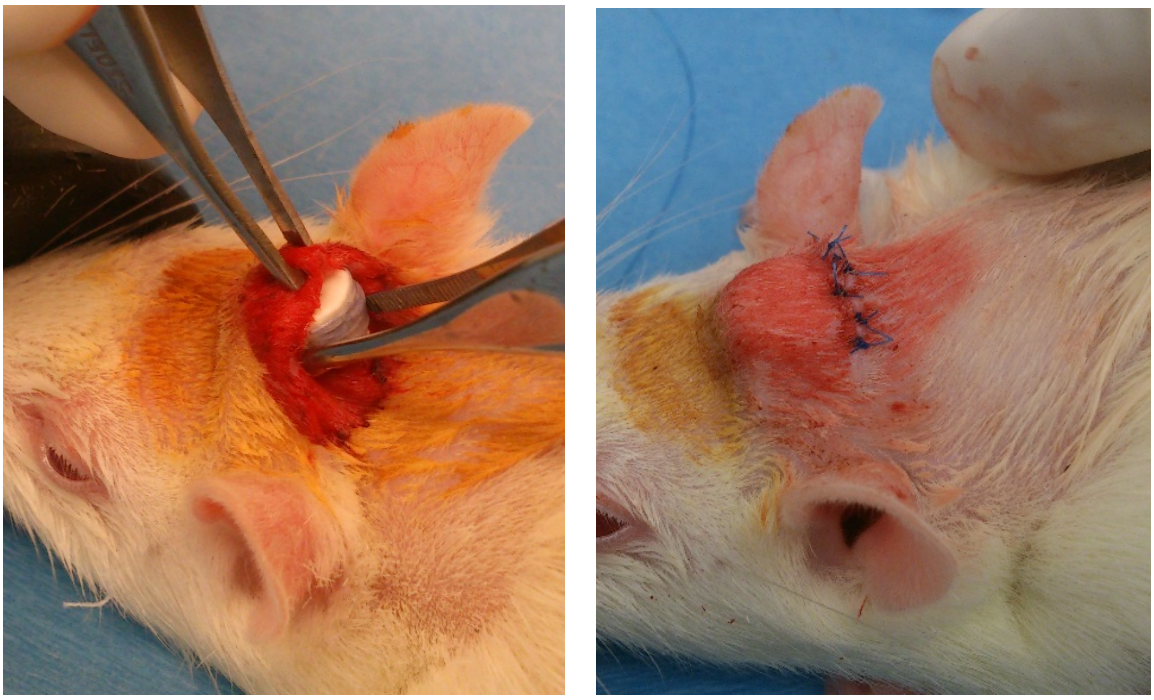
Figure 5.1: Fabrication of fast eroding and slow eroding multilayer device using metronidazole, ketoprofen, doxycycline and simvastatin loaded CAPP layers.

5.2.2 *In vitro* studies

The multilayered CAPP devices were eroded in phosphate-buffered saline (PBS), pH of 7.4, during incubation at 37ºC with gentle shaking. At regular time intervals, the supernatant was collected, the mass of the samples was measured, and fresh PBS was added. Sample masses were used to construct the mass loss profile of the multilayered devices. Supernatants were used to determine the amount of drug (metronidazole, ketoprofen, doxycycline, and simvastatin) using high performance liquid chromatography (HPLC; Hitachi Primaide) equipped with a C18 column (Kinetix; Phenomenex, Torrance, CA). For measuring the concentration of ketoprofen, an isocratic mobile phase of acetonitrile (60%) and 0.1% trifluoracetic acid (TFA; 40%) was used with UV detection at 260 nm, and for simvastatin, the isocratic mobile phase was acetonitrile (70%) and 0.1% TFA (30%) with UV detection at 240 nm was used. A gradient mobile phase with acetonitrile and 0.1% TFA was developed for measuring the concentrations of metronidazole and doxycycline with UV detection at 318 and 350 nm, respectively.

5.2.3 *In vivo* studies

All animal studies were conducted at the University of Kentucky in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC). Male Sprague-Dawley rats between the ages of 6 and 8 weeks were used. Four different types of devices were implanted: 1) blank, 2) high drug dose (5 wt%), 3) low drug dose (1 wt%), and 4) low drug dose with PSA blank layers for longer erosion time. After anesthetization and skin preparation, a transverse incision was made between the ears of the animal, and the periosteum was slightly elevated to expose the calvarium. CAPP devices were placed with the drug-releasing side facing the bone, and the incision was sutured (Figure 5.2). Animals were euthanized at specific time points (Table 5.1), and the devices were retrieved for analysis. Implants were cross-sectioned using a razor blade and imaged for morphological analysis (Figure 5.3). Residual mass and thickness of the CAPP layers present in the device were also measured. The retrieved devices were then completely dissolved in PBS, and the solution containing dissolved drugs and CAPP was filtered using a 0.45 μm syringe filter. This filtered solution was analyzed using same HPLC methods as described in the *in vitro* drug release section to determine the residual drug content.



(A) (B)

Figure 5.2: Implantation of a CAPP device over the rat calvarium. (A) Site of implantation during the surgery and (B) after closure.

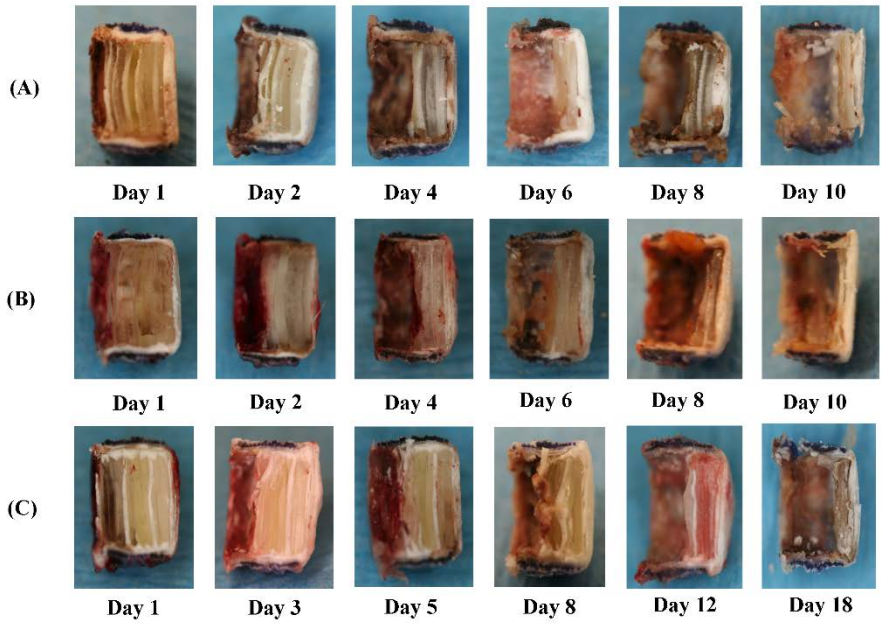


Figure 5.3: Cross-sectional images of devices retrieved following implantation for increasing durations. (A) High dose devices, (B) low dose devices, and (C) low dose devices with PSA blank layers.

Table 5.1: Time points at which samples were retrieved during the course of *in vivo* study (number of animals at each time point n=3)

Device type	Time points of device retrieval (days)												
High dose devices	**0.5**	**1**	**2**	**3**	**4**	**5**	**-**	**7**	**-**	**9**	**10**	**-**	**-**
Low dose devices	**-**	**1**	**2**	**-**	**4**	**-**	**6**	**-**	**8**	**-**	**10**	**-**	**-**
Low dose devices with PSA	**-**	**1**	**-**	**3**	**-**	**5**	**-**	**-**	**8**	**-**	**-**	**12**	**18**
Blank devices	**-**	**-**	**-**	**3**	**-**	**-**	**6**	**-**	**-**	**9**	**-**	**-**	**-**

5.2.4 Statistical analysis

Slopes of the *in vitro* and *in vivo* mass loss and thickness loss profiles were analyzed by linear regression using Graphpad Prism software. Differences were considered significant when $p<0.05$.

5.3 Results

5.3.1 *In vitro* mass loss profiles

Figure 5.4 shows mass loss profiles for the multilayered CAPP devices when eroded in PBS. Both types of CAPP devices, either with two blank layers or with a PSA layer between the two blank layers, followed similar linear erosion profiles. There was a statistically significant difference between the slopes of the mass loss curves during the course of the study ($p<0.0001$). Mass loss occurred at a faster rate for devices with just two blank layers when compared with devices also having the PSA layer. Mass loss occurred at a rate of 0.04 mg/mm^2/hour for 90:10 CAPP layers when sink conditions were maintained by replacing PBS at regular time intervals.

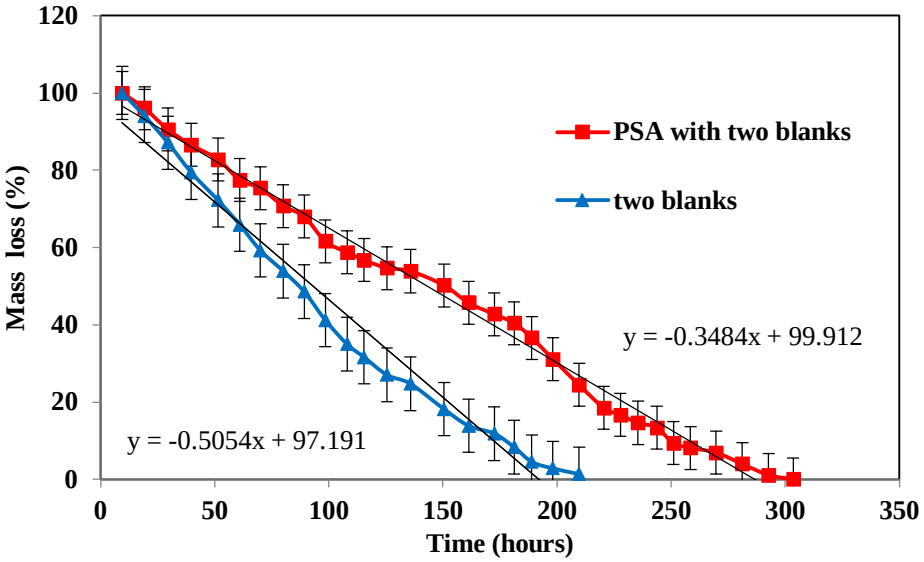
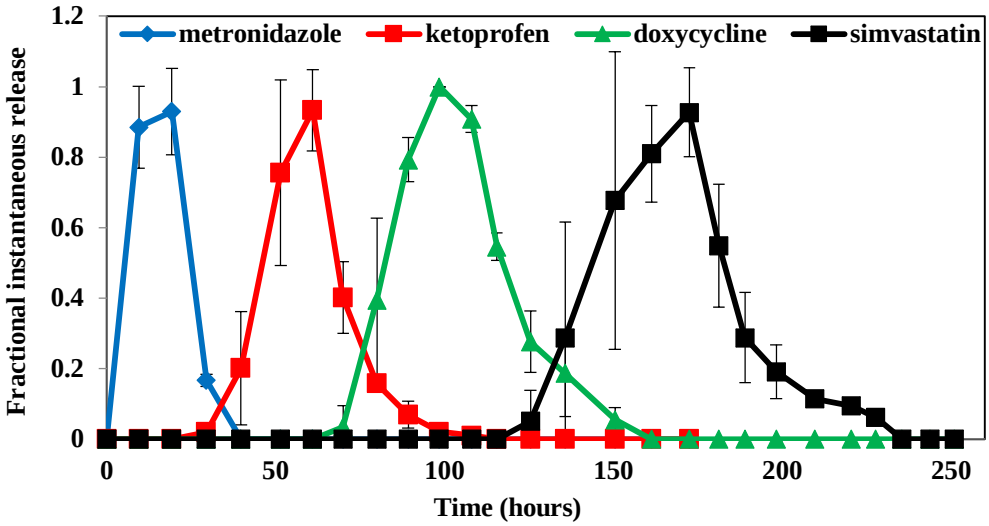


Figure 5.4: *In vitro* mass loss of 90:10 CAPP devices with two blanks (y = -1.229x + 236.35) and 90:10 CAPP devices with PSA layer between the blank layers (y = -0.8823x + 253).

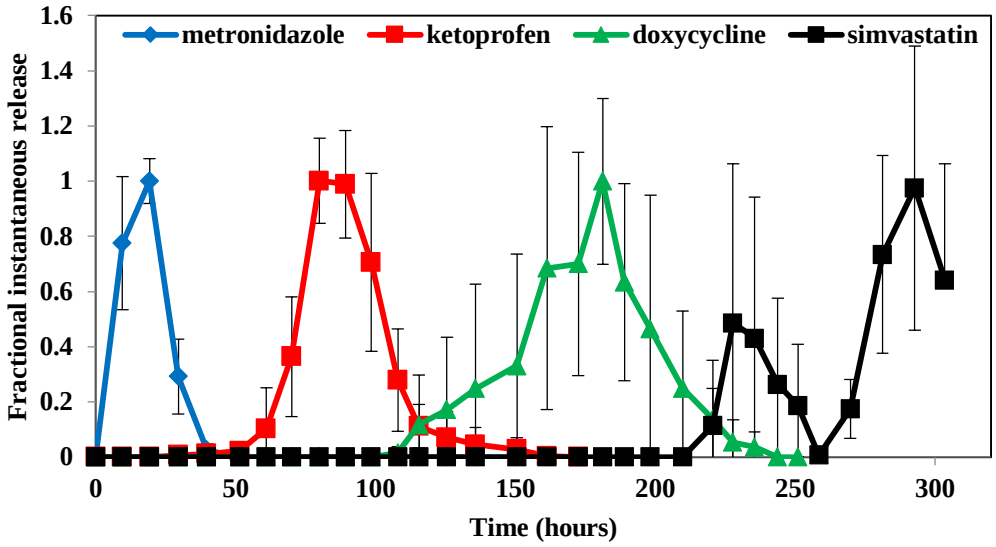
5.3.2 *In vitro* drug release profiles

Figure 5.5 A shows the instantaneous profiles for four drugs released from the multilayered delivery system with two blank layers. Because metronidazole was loaded in the outermost layer, it was released during the first 30-40 hours of incubation in PBS. This was followed by release of the anti-inflammatory drug ketoprofen from 40-90 hours during

the course of further device erosion. The third drug released from the system was doxycycline, which started around 70 hours and lasted through 150 hours. The last drug, simvastatin, was released during the final stages of device erosion (125-240 hours).



(A)



(B)

Figure 5.5: Sequential drug release from *in vitro* studies (A) 90:10 CAPP devices and (B) 90:10 CAPP devices with PSA layer between the blank layers.

The use of the PSA between the blank CAPP layers further increased the erosion time of devices to 300 hours. Release of metronidazole occurred within the first 40 hours, as in the case of devices without PSA layer, but the presence of PSA layers before the other drug layers slowed erosion and extended the release periods of other three drugs. Release of ketoprofen occurred through 125-135 hours, which was close to half of the total erosion time of the device. Release of the third drug, doxycycline, started at around 115 hours and lasted until 235 hours, and this was followed by simvastatin from 220 hours through complete erosion of the devices at around 300 hours. The times at which the peaks were observed *in vitro* for all four drugs in the two different device types are summarized in Table 5.2.

Table 5.2: Time of metronidazole, ketoprofen, doxycycline, and simvastatin peaks for the two types of devices fabricated and tested.

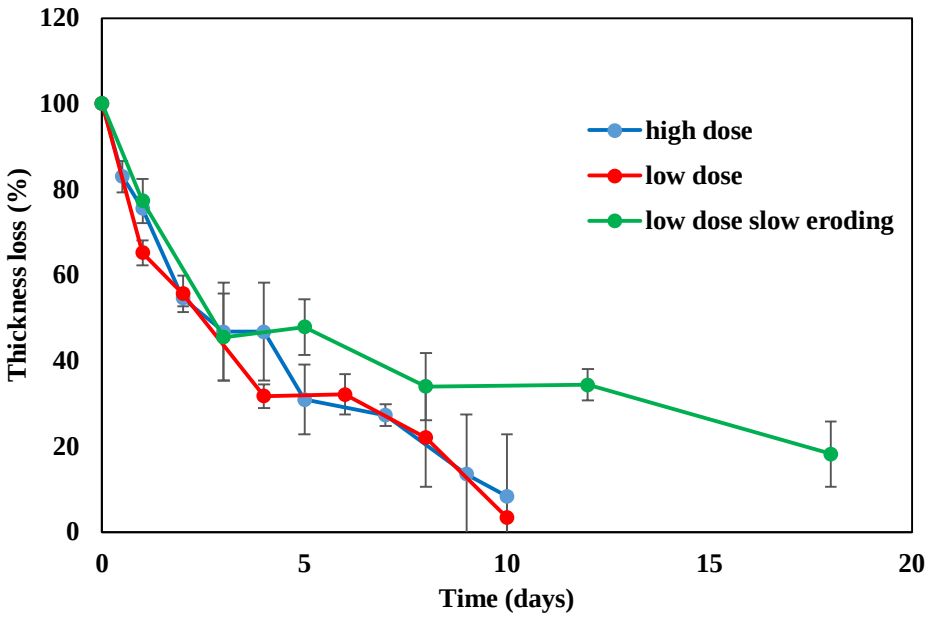
Device type	Metronidazole (hour)	Ketoprofen (hour)	Doxycycline (hour)	Simvastatin (hour)	Total erosion time (hours)
Two blanks	19	61	99	173	235
PSA with two blanks	19	90	181	293	300

5.3.3 *In vivo* thickness and mass loss profiles

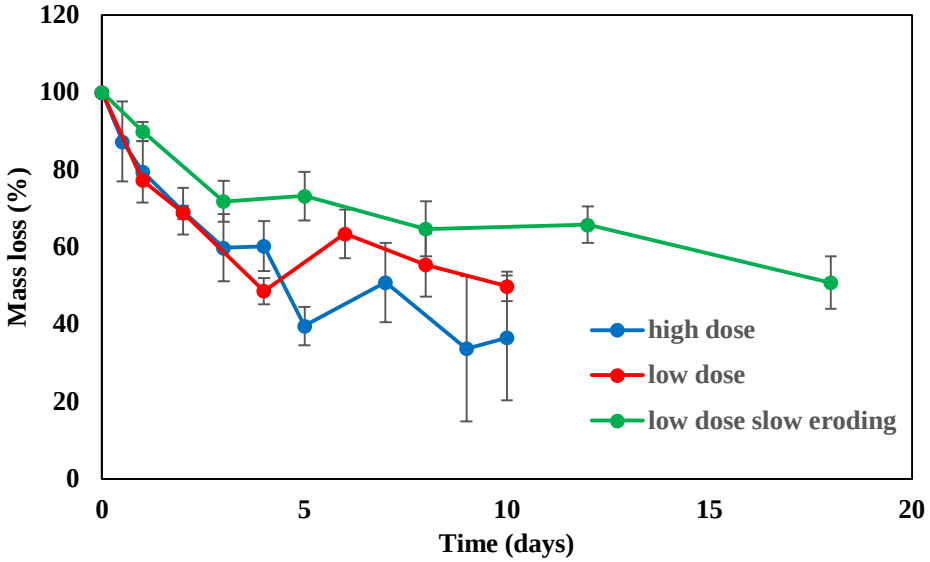
Cross-sections of the retrieved devices from all three groups (high dose, low dose, and slow-eroding low dose) showed gradual reduction in thickness of the CAPP layers (Figure 5.3). The high and low dose groups with two blank layers followed a similar loss in thickness, with almost all the CAPP layers eroded by day 10. The slower-eroding, low dose group with PSA layers eroded at a slower rate and had CAPP layers present in them even at day 18. Figure 5.6 A shows the thickness loss profiles for the three types of devices over the course of study. The high and low dose devices with two blank layers followed a biphasic pattern with thickness loss occurring at a faster rate until day three or four (approximately loss of first four layers, which includes the metronidazole and ketoprofen layers) followed by a slower rate of thickness loss until complete erosion by day 10. There was no statistically significant difference between the thickness loss profiles of the high and low dose groups. The slow-eroding, low dose group followed a similar biphasic

thickness loss pattern as observed in the case of faster-eroding devices, but at a slower rate with only 80% of thickness loss occurring by day 18. The slope of the thickness loss curve of the slow-eroding devices with PSA layers was significantly different from those for devices with just two blank layers ($p=0.04$).

The mass of devices measured after retrieval was used to construct the mass loss profiles shown in Figure 5.6 B. The high and low dose groups with two blank layers followed similar mass loss profiles, with no significant difference between the slopes of the curves. The slow-eroding low dose devices with PSA layers followed a mass loss profile but with a slower rate that was significantly different from that for devices with two blank layers ($p=0.002$).



(A)



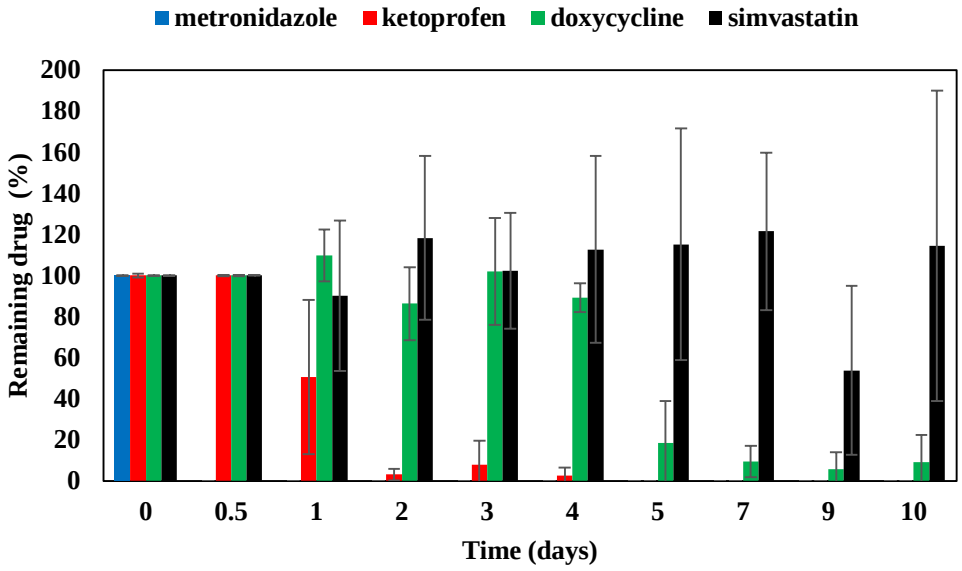
(B)

Figure 5.6: *In vivo* (A) thickness and (B) mass loss of high dose 90:10 CAPP devices, low dose 90:10 CAPP devices, and low dose slow eroding 90:10 CAPP devices with PSA layer between the blank layers.

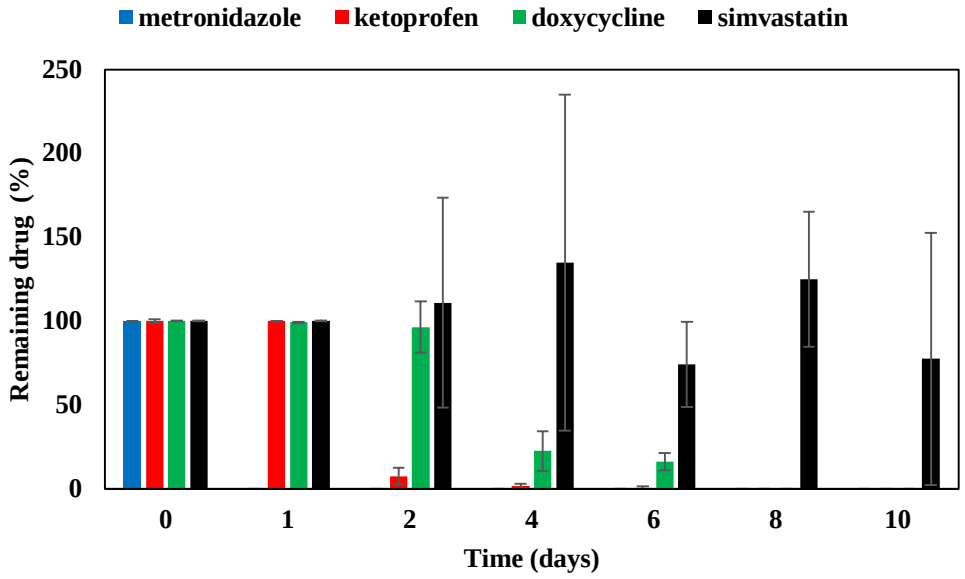
5.3.4 *In vivo* drug release profiles

HPLC measurement of the amount of drug present in the retrieved device was used to construct *in vivo* drug releases profile based on the percentage of different drugs remaining in the retrieved devices. For high dose devices (Figure 5.7 A), metronidazole was essentially gone at 12 hours, but 100% of the other three drugs was present. After one day of implantation, the retrieved devices showed about 50% of ketoprofen present and nearly 100% of doxycycline and simvastatin. There was no significant amount of ketoprofen observed after the second time point. The presence of doxycycline in the retrieved implants was observed until day 4. Approximately 100% of the simvastatin was found in the retrieved devices until the final stages of the study (day 9-10). In the case of low dose devices, a similar type of release pattern was observed (Figure 5.7 B), with metronidazole completely released by first time point (24 hours) and nearly 100% of the other three drugs still present in the implant. Implants retrieved at the second time point (day 2) showed retention of 100% doxycycline and simvastatin, with ketoprofen completely released with the exception of some trace amounts. There were traces of doxycycline observed in the remaining time points, with simvastatin measured in the retrieved devices until the last time point.

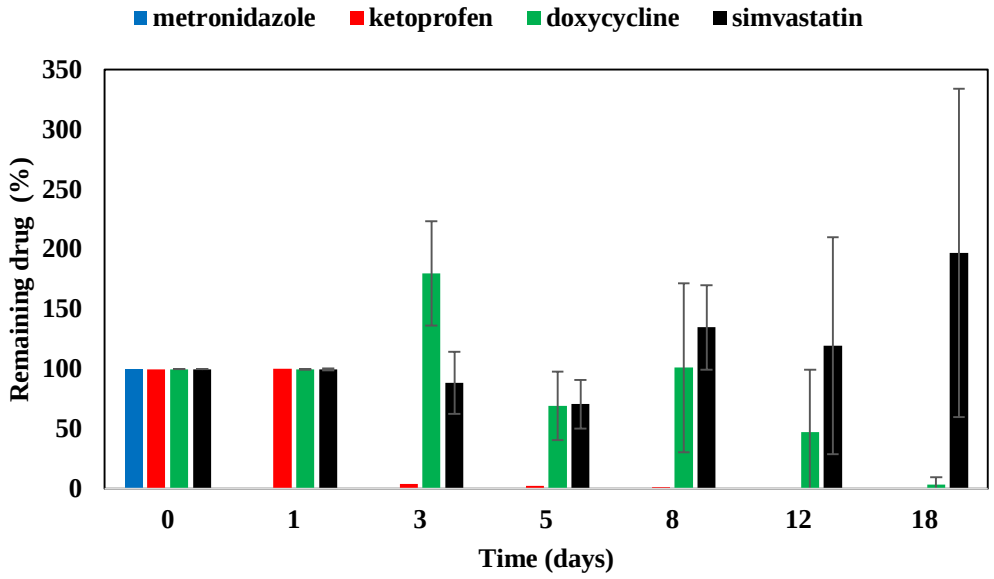
Devices with intermediate PSA layers followed a sequential release pattern of four drugs but at a slower rate (Figure 5.7 C). Similar to the high and low dose devices, metronidazole was completely released within 24 hours. Ketoprofen was observed in the devices until day 3 and traces observed in devices retrieved on day 5. Through day 12, nearly 100% of doxycycline was found in the retrieved devices, and at day 18 (last time point) approximately 50% of the doxycycline was observed. With the devices not being completely eroded by day 18, almost 100% of simvastatin was observed in all the retrieved devices. Release of the last drug (simvastatin) was observed to be more delayed in all the three types of devices when compared with the other three drugs released. Table 5.3 gives an overview of the time points through which the specific drugs were observed *in vivo* for the different types of devices.


metronidazole
ketoprofen
doxycycline
simvastatin
Remaining drug (%)
200
180
160
140
120
100
80
60
40
20
0
0
0.5
1
2
3
4
5
7
9
10
Time (days)

(A)


metronidazole
ketoprofen
doxycycline
simvastatin
Remaining drug (%)
250
200
150
100
50
0
0
1
2
4
6
8
10
Time (days)

(B)



(C)

Figure 5.7: Sequential drug release from *in vivo* studies: (A) high dose devices, (B) low dose devices, and (C) low dose devices with PSA layers.

Table 5.3: Comparison of *in vitro* and *in vivo* release of metronidazole, ketoprofen, doxycycline, and simvastatin, indicating the time points through which the release of particular type of drug occurred for the specific types of devices. Note: times given in hours.

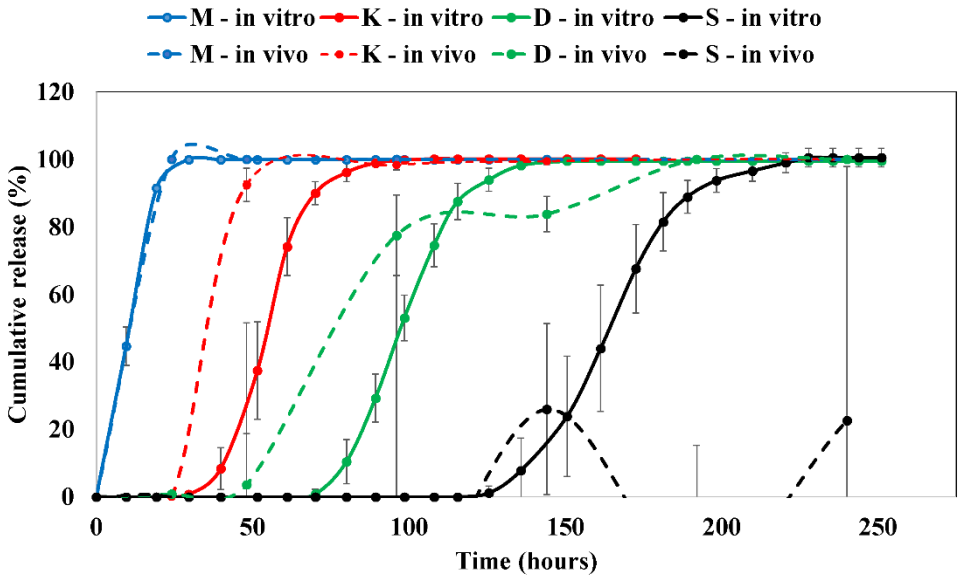
Drug type	Faster eroding devices			Slower eroding devices	
	In vitro	*in vivo* (high dose)	*in vivo* (low dose)	*In vitro*	*in vivo*
Metronidazole	30	≤12	≤24	30	≤24
Ketoprofen	90	72-96	72-96	135	120
Doxycycline	150	144-180 (with traces observed in later time points)	144-180 (with traces observed in later time points)	240	432
Simvastatin	235	>240	>240	300	>430

5.3.5 Comparison of *in vitro* and *in vivo* drug release profiles

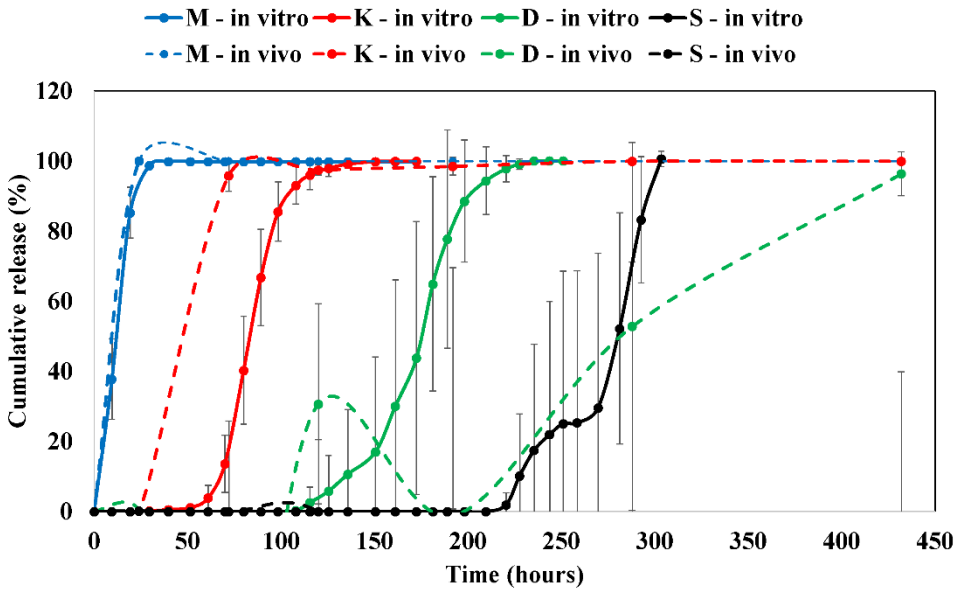
In vitro and *in vivo* release profiles for devices with two blank CAPP layers are compared in Figure 5.8A. For these faster-eroding devices, metronidazole release (from the first layer exposed) was completed within the first 12-24 hours *in vitro* and by 30 hours *in vivo*. Ketoprofen release *in vivo* occurred slightly faster than *in vitro*; in both the cases, ketoprofen release was complete within 90-100 hours. For the third drug, doxycycline, *in vivo* release occurred at a slightly slower rate when compared to that of the *in vitro* release. Doxycycline was completely released by 150 hours *in vitro*, compared to release of 80-90% at this time *in vivo*. Simvastatin release (from the last layer) *in vivo* was much slower than under *in vitro* conditions. By 240 hours, simvastatin was completely released *in vitro*, but simvastatin layers remained in the devices retrieved at 10 days of implantation.

Figure 5.8B compares *in vitro* and *in vivo* cumulative release profiles for devices with PSA layers that slow erosion. Similar to the faster-eroding devices, metronidazole release also occurred within 24hours for the slower-eroding devices under both conditions. Release of ketoprofen again occurred faster *in vitro* when compared to *in vivo*. It took approximately 150 hours for complete release of ketoprofen from slower-eroding devices *in vitro*, whereas 90% of the drug was released by 72 hours from slower-eroding devices *in vivo*. Similar to the faster-eroding devices, doxycycline (third drug) release *in vivo* was slower than its *in vitro* release. Only 53% of doxycycline was released *in vivo* by 288 hours compared to complete release *in vitro* by 240 hours. Simvastatin release from slow-eroding devices *in vitro* was completed by 300 hours, but all devices retrieved at day 18 (last time point) had the complete simvastatin layer still present, indicating that simvastatin release had not yet started before 432 hours. Table 5.3 summarizes the temporal differences

between the faster- and slower-eroding devices *in vitro* and *in vivo* based on the time points through which release of a particular type of drug occurred.



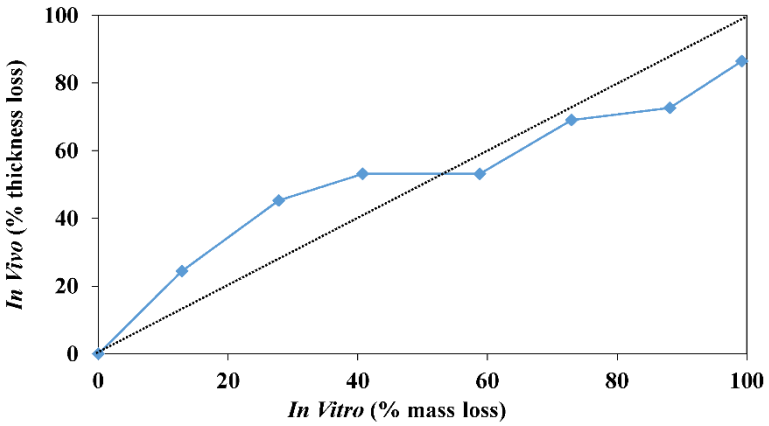
(A)



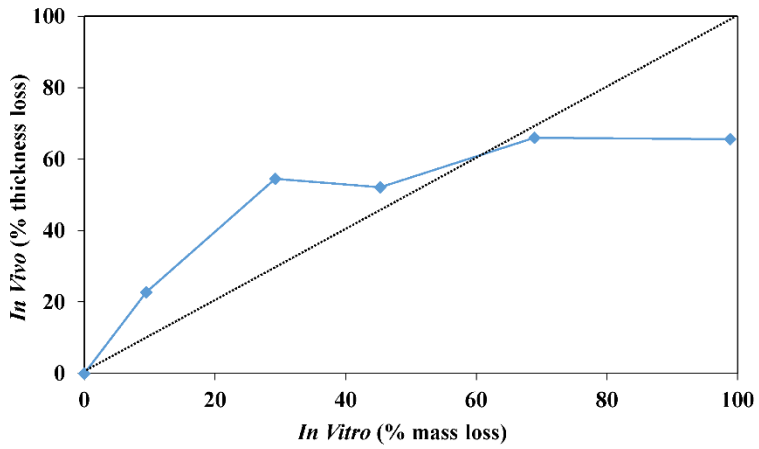
(B)

Figure 5.8: Comparison of *in vitro* and *in vivo* cumulative drug release of metronidazole (M), ketoprofen (K), doxycycline (D) and simvastatin (S) from (A) faster and (B) slower eroding multilayered CAPP devices.

Figure 5.9 directly compares *in vitro* mass loss and *in vivo* thickness loss for the fast- and slow-eroding devices. *In vivo* thickness loss was used instead of mass loss because blood and tissue adherent on retrieved devices made *in vivo* mass measurements inaccurate. These plots show the increased erosion rate during the initial stages and relatively slower erosion rate that was observed during the final stages for both types of devices *in vivo*.



(A)



(B)

Figure 5.9: Comparison of *in vitro* mass loss and *in vivo* thickness loss of (A) faster and (B) slower eroding multilayered CAPP devices.

5.4 Discussion

Multilayered devices based on the CAPP association polymer system have demonstrated the ability to deliver different types of drugs in a required sequence *in vitro* (Sharath C. Sundararaj 2013). Previous research involving this system was conducted using a CAP:P ratio of 70:30. In this particular study, the system was modified to have a ratio of 90:10 to achieve longer erosion times. The main aim of this research was to investigate the performance of this CAPP system under *in vivo* conditions and relate the findings to what is seen with standard *in vitro* release experiments. Pilot *in vivo* studies (data not shown) showed that devices with the CAP:P ratio of 90:10 had longer erosion time when compared to devices fabricated using 70:30. These *in vivo* data were comparable to the present *in vitro* data, which showed that the 90:10 CAPP devices (complete erosion time of 240 hours) had a two-fold increase in erosion time compared with similar devices made with 70:30 CAPP (complete erosion time of 120 hours) (Sharath C. Sundararaj 2013). Based on these *in vitro* and pilot *in vivo* results, multilayered devices were fabricated with 90:10 CAPP films for a larger scale study to compare *in vitro* and *in vivo* material responses. To achieve an even longer erosion time, PSA layers, which erode more slowly than does CAPP, was incorporated between the blank layers.

In addition to the primary component of CAPP, the surface-eroding polyanhydride PSA (Kipper, Shen et al. 2002) was a part of the delivery system. PSA was used as a barrier in all devices and as a blank layer in the slower-eroding devices. The rate of erosion of PSA is slower than that of CAPP, making it an appropriate material of choice for a barrier layer to achieve unidirectional erosion. Furthermore, the thin (approximately 300 µm) intermediate layers between the blank CAPP layers further decreased the erosion rate. The presence of three layers of PSA decreased the overall rate of mass loss, thereby increasing the total erosion time of the device with only a slight increase in total thickness of the device (< 1 mm) when compared to the devices with just two blank CAPP layers. The adjustable nature of the delivery system with respect to the erosion time creates the possibility of designing devices based on specific disease conditions.

In vitro mass loss and drug release

In vitro, devices with two blank layers showed sequential release of the four drugs metronidazole, ketoprofen, doxycycline, and simvastatin. As intended, slower-eroding

devices with PSA layers also showed sequential release of the four drugs, with better distinction between the drug release peaks compared to devices without PSA layers. Attachment of the final simvastatin-loaded CAPP layer to the PSA barrier might have affected the erosion rate of this particular layer, which resulted in the less uniform release of simvastatin compared to the other drugs. This can be resolved in future device fabrication by incorporating a thin blank CAPP layer between the simvastatin layer and PSA barrier; in this way, the drug layer and PSA barrier are separated, which should avoid non-uniform erosion and drug release. It was also observed that the simvastatin and doxycycline release peaks were broader and took more time to release. This could be because of the cylindrical, well-shaped design of the PSA barrier leading to retention of PBS within the barrier shell as the initial CAPP layers eroded, resulting in reduced circulation of fluid near the final drug layers leading to slower erosion of polymer and release of drugs.

In vivo thickness loss, mass loss, and drug release

The bottom CAPP layers were seen at day 18 in the case of devices with PSA layers compared to near complete erosion in devices with just CAPP layers by day 10. This observation shows that the PSA-containing devices retained their slower-eroding properties *in vivo*, as it was previously demonstrated *in vitro*. Quantitatively, the loss of thickness followed a pattern comparable to that seen qualititively of the findings observed in the cross-sectional images. The biphasic thickness loss patterns observed for both the high and low dose devices were not significantly different, indicating that the amount of drug loaded in the device did not have any effect on the erosion rate of the device. Thus, dosing can be adjusted for different future applications. Devices with PSA layers also followed a similar biphasic thickness loss pattern but at a slower erosion rate, demonstrating the ability of the PSA layers to slow down the erosion rate of the device without drastically altering the erosion pattern. Even though the presence of blood and tissue attached to the remaining CAPP layers and the PSA barrier of retrieved devices made it difficult to accurately measure mass, the pattern of mass loss observed was comparable to that of the thickness loss. The biphasic thickness and mass loss patterns could be mostly due to reduced circulation of fluid because of cylindrical well-based design of the PSA barrier layer as described in the previous *in vitro* mass loss and drug release section. Along

with the reduced circulation of fluid after the erosion of the initial CAPP layers, the ingrowth of fibrous tissues in to the well-shaped PSA shell could have further slowed erosion of CAPP layers in the deeper end of the devices *in vivo*.

Analysis of the drugs present in the retrieved devices indicated that the drugs were released in a step-by-step manner *in vivo*. For example, when the first drug, metronidazole, was completely released, the other three drugs were still present in the device. Importantly, the other drugs were not released by diffusion. This type of behavior was observed for the rest of the four drugs. For all three types of devices, *i.e.*, high dose, low dose, and slow-eroding, drug release occurred in the intended sequential manner *in vivo*. This indicates that the dose of the drug or the rate of device erosion did not have a substantive effect on the sequential drug release pattern. Even *in vivo*, the CAPP layer loaded with simvastatin attached to the PSA barrier resulted in non-uniform erosion and drug release as observed *in vitro*. The extended release of doxycycline and simvastatin relative to the release of first two drugs, metronidazole and ketoprofen, was comparable to the *in vitro* release of these drug in the particular sequence. This also corresponds with the biphasic mass loss and thickness loss that was observed *in vivo*.

Comparison of in vitro and in vivo performance

On the whole, devices initially eroded and released drugs more quickly, but during the later stages, the device design and physiological conditions played major roles in reducing the rates of polymer erosion and drug release when compared to their *in vitro* behaviors. The faster-eroding devices are predicted to last 270-300 hours and the slower eroding device for 500-600 hours *in vivo*. Formation of fibrous tissue, observed in the devices retrieved after one week of implantation, combined with the previously described retention of fluid within the cylindrical, cup-shaped PSA barrier appeared to have slowed the clearance of erosion byproducts and the consequent erosion-dependent release of drugs. These effects were more distinct in the case of slower-eroding devices.

Along with differences in overall erosion rate *in vitro* and *in vivo*, the release rate of specific drugs was affected by their position in the multilayered device. The first two drugs were released faster and the last two drugs were released more slowly *in vivo*, when compared to their corresponding *in vitro* release profiles. This comparative information

could be valuable for precisely designing devices capable of releasing multiple drugs in a predetermined sequential order. For example, the particular drugs selected make the system relevant for potential treatment of periodontitis (Sharath C. Sundararaj 2013). Knowing the effect of implantation on the erosion and release profiles enables "tuning" the system to deliver the active agents at a particular time. The *in vivo* results clearly suggest that the last drug released from the device would be delayed when compared to the corresponding *in vitro* release from the same device. Based on this, the PSA layer before the simvastatin layer can be removed to counter the delay that occurs *in vivo* or the blank CAPP layers can be replaced by thicker PSA layers to further delay the release if required.

Comparisons of *in vivo* and *in vitro* drug release have generally been conducted for delivery systems containing a single drug, with the *in vivo* drug release calculated by the measuring the amount of drug in the plasma or by measuring the amount of drug remaining in the device/delivery system (Schmidt, Wenz et al. 1995) (Avgoustakis, Beletsi et al. 2002, Mittal, Sahana et al. 2007, Zolnik and Burgess 2008). Based on the type of polymer used, type of delivery system, and the physiological effects on the device, release profiles are similar both *in vitro* and *in vivo* in some cases (Schmidt, Wenz et al. 1995). In others, *in vitro* release was different from what was measured *in vivo* (Zolnik and Burgess 2008). The present study involved a *in vivo*/*in vitro* comparison of drug release for a more complex delivery system involving sequential release of multiple (four) drugs. The comparison indicates a difference between the *in vitro* and *in vivo* drug release profiles. Such information can be useful for designing complex multiple drug delivery systems and understanding how they are affected by physiological conditions.

5.5 Conclusions

Multilayered, bioerodible CAPP devices were successful in delivering four drugs in a sequential manner both *in vitro* and *in vivo*. Polymer erosion rate can be adjusted to shift release peaks, and drug dose can be changed while maintaining the temporal nature of the profile. Although the overall time course was longer *in vivo*, erosion and release were initially faster but then slowed in comparison to standard *in vitro* conditions. This type of comparative information will be useful for modifying the delivery system to obtain programmed sequential release profiles.

Chapter 6 Conclusion

A CAPP association polymer based device has been developed, capable of releasing multiple drugs by in a predetermined temporal sequence. The initial studies showed that the CAPP association polymer fabricated in the form of films can be used to load and release different types of small molecule drugs and a model protein primarily by surface erosion. Bioactivity analysis of the released drugs showed the complete retention of bioactivity in small molecule drugs. The device fabricated in the form of multilayered CAPP films was able to release the same drug in an intermittent fashion and two different types of drugs in a sequential order. Even though the CAPP multilayer device was designed for releasing different types of drugs sequentially in any required order for treatment of complex disease conditions and tissue engineering purposes, this device was specifically modified for sequential release of four drugs for stepwise treatment of periodontitis. The device was capable of releasing antibiotic, anti-inflammatory, anti-resorptive and osteogenic agents in a temporal sequence aimed at the treatment of infection, inflammation, tissue loss and alveolar bone regeneration stages of periodontal disease. Different erosion times of the devices were obtained by changing the CAP:Pluronic ratio from 70:30 to 90:10 and by inclusion of a slower eroding PSA layer in between the CAPP layers. This would make it possible to change the erosion time of the device, thus altering the drug release rates based on the requirement of the condition. The sequential release of these four drugs has also been successfully demonstrated *in vivo*. Devices loaded with both high and low doses of drugs followed sequential release pattern in a supracalvarial implantation site, indicating the possibility of achieving different drug doses depending on the severity of the condition. Comparison of the *in vitro* and *in vivo* drug release profiles provided insights on the possible changes in the device that might be required for its use in the treatment in animal model. Future work would involve the modification of this device based on the *in vivo* data obtained for treatment in a periodontal animal model and exploring other possible tissue engineering and treatment application which might need this type of sequential multiple drug delivery system.

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EDUCATION

University of Kentucky, Lexington, Kentucky, 2010
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SRM University, Chennai, India, 2007
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RESEARCH EXPERIENCE

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AWARDS:

- Research on Multiple Drug Delivery system aimed at the Treatment of Periodontitis nominated as an honorable mention for outstanding contribution to the Society For Biomaterials 2012 Fall Symposium. (2012)

- Received Max Steckler Fellowship.(2011-2012)

- Recipient of Kentucky Graduate Scholarship.(2007-2010)

PUBLICATIONS:

- Sundararaj SC, Al-Sabbagh M, Thomas MV, Dziubla TD, Rabek CL and Puleo DA. "Comparison of *in vitro* and *in vivo* sequential drug release" in preparation for submission. *Journal of Controlled Release*. (2013)

- Sundararaj SC, Peyyala R, Thomas MV, Dziubla TD and Puleo DA. "Design of a Multiple Drug Delivery System Directed at Periodontitis" *Biomaterials.* (2013) 34(34):8835-42.

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- Sundararaj SC, Cieply RD, Gupta G, Milbrandt TA, and Puleo DA. "Treatment of Growth Plate Injury Using IGF-I Loaded PLGA Scaffolds" *The Journal of Tissue Engineering and Regenerative Medicine*. (2012) in press

- Medley JM, Kaplan E, Oz HS, Sundararaj SC, Puleo DA and Dziubla TD. "Fibrin Targeted Block Copolymers for the Prevention of Postsurgical Adhesions" *J Biomed Mater Res Part B.* (2011) 99B:102–110.

- Medley JM, Beane EJ, Sundararaj SC, Kaplan E, Puleo DA and Dziubla TD. "Block copolymers for the rational design of self-forming postsurgical adhesion barriers" *Acta Biomaterialia*. (2010) 6(1):72-82.

PROFESSIONAL CONFERENCE PRESENTATIONS (POSTER/ORAL):

- Sundararaj, S.K.C., Thomas, M.V., Al-Sabbagh, A., Dziubla, T.D., and Puleo, D.A. "Sequential drug delivery – *in vitro* and *in vivo"*. Presented at the Annual Meeting of the Society For Biomaterials, April 2013, Boston, MA.

- Sundararaj, S.K.C., Thomas, M.V., Dziubla, T.D., and Puleo, D.A. "Sequential drug delivery aimed at treatment of periodontitis". Presented at the Annual Meeting of the Society For Biomaterials, April 2012, New Orleans, Louisiana.

- Sundararaj, S.K.C., Thomas, M.V., Dziubla, T.D., Peyyala R., and Puleo, D.A. "CAP-Pluronic Film Based Multiple Drug Delivery System for treatment of periodontitis" Presented at the American Association for Dental Research Annual Meeting, March 2012, Tampa, Florida, USA.

- Sundararaj, S.K.C., Thomas, M.V., Dziubla, T.D., and Puleo, D.A. "CAP-Pluronic Film Based Multiple Drug Delivery System for treatment of periodontitis" Presented at the Society For Biomaterials Annual Meeting and Exposition, April 2011, Orlando, Florida, USA.

- Sundararaj, S.K.C., Thomas, M.V., Dziubla, T.D., and Puleo, D.A. "Multiple drug delivery system based on CAP-Pluronic association polymer" Presented at the Biomedical Engineering Society annual meeting, October 2010, Austin, Texas, USA.

- Sundararaj, S.K.C., Milbrandt T., Hilt Z., and Puleo, D.A. "Tissue Engineering of Growth Plate using IGF-I Releasing PLGA Scaffolds" Presented at the Society For Biomaterials Annual Meeting and Exposition, April 2010, Seattle, Washington, USA.

- Sundararaj, S.K.C., Milbrandt T., Hilt Z., and Puleo, D.A. "Chondrogenic Growth Factor Releasing Scaffolds for Regeneration of Growth Plates" was presented at the Annual meeting and Exposition, April 2009, San Antonio, Texas, USA.