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MUCOADHESIVE FILMS FOR TREATMENT OF LOCAL ORAL DISORDERS: DEVELOPMENT, CHARACTERIZATION AND *IN VIVO* TESTING

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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

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ABSTRACT OF THIS DISSERTATION

MUCOADHESIVE FILMS FOR TREATMENT OF LOCAL ORAL DISORDERS: DEVELOPMENT, CHARACTERIZATION AND *IN VIVO* TESTING

Mucoadhesive drug delivery systems which are being used from 1980's to avoid first pass metabolism of drugs, commercially exist for only systemic drug delivery with fast erosion times (15-60 min), that may not be appropriate for local oral disorders. The goal of this research was to develop and characterize mucoadhesive films with flexibility of carrying different drugs and proteins and provide sustained release for local treatment of oral disorders.

Mucoadhesive films composed of polyvinylpyrrolidone (PVP) and carboxymethlycellulose (CMC) were formulated with imiquimod, an immune response modifier. Problems such as solubilization of imiquimod to increase drug loading, uniformity in films and total amount of drug released into supernatants were addressed by use of acetate buffer after investigating multiple methods.

Subsequently, other relevant properties of mucoadhesive systems, such as adhesion (shear, pull-off), tensile properties, swelling profiles, transport kinetics, and subsequent changes in release profiles as a function of film composition were characterized. The potential of the system for local retention of imiquimod, determined in oral mucosa of hamsters showed time dependent decrease in imiquimod amount through 12 hours, with no traces of drug in blood. Further testing in humans revealed that the residence time of the mucoadhesive films depended on the application site, increasing in the order of tongue < cheek < gingiva.

In parallel, mucoadhesive films loaded with epidermal growth factor (EGF) were developed to promote treatment of oral mucosal wounds. Bioactivity was tested *in vitro* on buccal tissues by creating a wound followed by application of films. Although EGF-loaded films did not accelerate wound healing, but rather elicited a hyperparakeratotic response. In vitro buccal tissues may not be appropriate for testing the effects of EGF in wound healing without incorporation of other biochemical factors.

Overall, a mucoadhesive system capable of delivering bioactive small molecules and proteins in sustained manner was developed in this work. A thorough understanding of the system properties was achieved to further tune for future applications. *In vitro* studies and *in vivo* studies in hamsters and humans clearly showed the potential and usefulness of the system to translate in to clinic for treatment of oral precancerous lesions.

KEYWORDS: Mucoadhesive films, imiquimod, oral dysplasia, mucosal wound healing, local treatment.

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Chapter 1 Introduction

Oral mucosal membranes composed of epithelial cell layers and connective tissue act as an efficient semi-permeable barrier system, allowing diffusion of water, nutrients, gases, and small molecules, while remaining impermeable to bacteria and pathogens. Delivery of drugs through the oral mucosa has gained prominence in the last two decades because of its rich vasculature, which enables rapid delivery and onset of action and avoids first pass metabolism of drugs (1, 2).

Mucoadhesive formulations dating back to 1947 have significantly improved from 1980s in delivery of active molecules to various mucosal surfaces (3). Different formulations such as gums, films, tablets, gels, and microparticles were developed to deliver molecules via oral transmucosal route (2). These systems adhere to the mucosal surfaces and provides high flux of drug transport, increases bioavailability, improves permeability, and protects the structure of proteins (4). Commercially approved mucoadhesive drug delivery systems (Striant™, Nitrogard®, Fortfivo XL^R, etc) are being used to treat systemic disorders only. In this dissertation, a mucoadhesive system composed of polyvinylpyrrolidone (PVP) and carboxymethylcellulose (CMC) was developed to treat local disorders such as oral dysplasia and mucosal wound regeneration.

Oral squamous cell carcinoma (OSCC), estimated to effect 42,440 patients in 2014, is preceded by abnormally matured precancerous lesions known as oral dysplastic lesions. Treatment of these precancerous lesions can prevent them from progressing to OSCC and avoid further complications (5, 6). Imiquimod, an immune response modifier in form of Aldara® cream, was successful in treatment of the superficial basal cell carcinoma and few clinical cases of oral leukoplakia (7, 8). Hence, as described in chapter 3, a bilayered mucoadhesive film loaded with imiquimod was fabricated by solvent casting method. Because of highly hydrophobic nature of imiquimod, three methods of loading were also investigated to increase solubility and loading capacity of the films. Mucoadhesiveness of the films and bioactivity of entrapped imiquimod were confirmed before further characterization and development.

Five types of films, including the former one were then fabricated by simply changing the composition of PVP and CMC, and characterized in hopes of identifying a formulation with all desired properties. Key factors for successful bioerodible mucoadhesive system such as tensile properties, adhesion strength, and swelling rates were characterized and compared in chapter 4. Two compositions were shortlisted and further compared based on their release profiles and erosion characteristics. Interestingly, permeability studies of the films on porcine mucosal tissue revealed significant increase in localization of imiquimod within epithelium compared to drug solution.

The performance characteristics of the mucoadhesive films were investigated *in vivo* in chapter 5. Residence time of the films were determined at three different application sites in humans to get an overall assessment. Because of no abundant literature about testing of long residing mucoadhesives in small animal models, the hamster cheek pouch model was investigated to determine the appropriate site for testing of the films. The localization of imiquimod in mucosal tissue as observed in permeability studies was verified and quantified in hamsters, and compared to commercial imiquimod cream (prescribed for skin lesions) in this chapter.

The versatility of the current delivery system to load a variety of drugs, such as small molecules and proteins, was shown in chapter 6, by fabricating epidermal growth factor (EGF) and lysoszyme loaded films. These films are then used to address another local disorder, mucosal wound healing. The films achieved sustained release of bioactive EGF for up to 6 hours *in vitro*. The efficacy of the films was tested on *in vitro* buccal tissues (ORL 300-FT) by making a wound of 3 mm in diameter.

With a vision of addressing more disorders in the future and improve treatment processes, various attempts to increase residence time of the films were discussed in the appendix of this dissertation. Addition/substitution of new polymers to the existing polymer composition was investigated to improve the erosion time and prolong drug release. Although some properties were improved in these studies, the enhancement of all essential properties was not achieved. In addition to new polymers, other changes such as design of the system, and specific adhesions are desired in order to achieve balance in improving properties.

Chapter 2 Background

2.1 Biology of Oral Mucosa

2.1.1 Structure

Oral mucosa is a mucus membrane, lining the majority of tissues in the oral cavity, such as lips, gingivae, and palate. Oral mucosa is made up of two layers: surface stratified squamous epithelium and underlying connective tissue, lamina propria. Epithelium protects underlying tissues by acting as a mechanical barrier, and also absorbs nutrients and various compounds. Lamina propria, which acts as a mechanical support to epithelium, comprises of collagen, elastic fibers and cells in an aqueous ground substance. Lamina propria also contains lymph system, nerves, and a rich supply of blood vessels that helps in fast transportation and clearance of absorbed molecules. (9).

Stratified squamous epithelium is composed of three distinct epithelial cell layers: superficial keratinized/non keratinized squamous layer, intermediate spinous cell layer, and basal cell layer. Active basal cells derive nutrients from lamina propria in order to mature, change shape, and differentiate, while advancing through the intermediate layers to the superficial layers (2, 10). The epithelium is supported by an underlying basement membrane that provides the required adherence between the epithelium and lamina propria.

Oral mucosa with a surface area of 200 cm^2, varies widely in terms of thickness and keratinization depending on the region of the oral cavity. It is divided into three types based on its structure. 1) Masticatory mucosa, constituting 25% of total oral mucosa covers the gingivae and hard palate. It is keratinized and closely resembles epidermis of the skin. 2) Lining mucosa (60%), is non-keratinized and generally thicker than masticatory mucosa, and covers buccal, sublingual and inner side of lips. 3) Specialized mucosa, (15%) which covers the top surface of the tongue, comprises properties of both masticatory and lining mucosa (9).

2.1.2 Mucus

Epithelium is coated and surrounded by a heterogeneous viscoelastic gel called mucus. It plays a role in cell-cell adhesion, relative motion of cells, and adhesive properties of the oral mucosa (2). While this intercellular substance is produced by special goblet cells at other mucosal regions, it is produced by major and minor salivary glands in the oral cavity. pH of mucus coating in the oral cavity ranges from 6.2 to 7.4 with thickness ranging from 40 to 300 µm. Mucus is composed of 95% of water and other constituents such as inorganic salts (1%), carbohydrates and lipids (1%), and glycoproteins (<5%) (11). Glycoproteins, also known as mucins, are responsible for gel and viscoelastic properties of mucus. Mucins are made up of 500 kDa subunits that are oligosaccharide based graft chains on protein-based backbones. These subunits are linked together by peptide linkages and/or intra molecular cysteine-cysteine disulfide bridges to form mega chains ranging from 1 kDa to 40 MDa (11).

Oligosaccharides which constitute 80% of the total weight of mucin, covers 63% of the length of protein backbone. The amino acid composition of protein backbone is dominated by 70% of serine, threonine, and proline. Oligosaccharides chains are composed of sugar residues N-acetylgalastosamine, N-acetylglucosamine, galacotose, fructose and sialic acid (3). Presence of carboxylate groups and ester sulfate groups in the sugar residues resulted in a net negative charge with pK_a of 1.0 to 2.6, and attracts water. This water attracting properties of oligosaccharides make mucins hydrosoluble and protect protein from proteolytic degradation. Because of several possible interactions of proteins and sugar residues with polymers, mucus is responsible and advantageous for maintaining adhesion of mucoadhesive formulations. However, short turnover life of mucus hinders prolonged residence time of dosage forms.

2.2 Drug Delivery to Oral Mucosa

Oral mucosal membranes act as an efficient semi-permeable barrier system allowing diffusion of water, nutrients, gases and small molecules, while remaining impermeable to bacteria and pathogens. High vasculature in the connective tissue of oral mucosa provides direct diffusion of permeated molecules in to the systemic circulation. This avoids first pass metabolism of drugs and can cause rapid onset of their action. Permeability of active compounds varies with regions of the oral cavity and is inversely proportional to keratinization and thickness of tissue. Molecules are most permeable through sublingual regions, followed by buccal and palate surfaces. Although sublingual regions are advantageous for rapid onset delivery because of high blood flow and less permeability, they are not suitable for controlled release systems because of constant saliva washing and tongue activity, which makes it difficult for dosage forms to reside on surface. In contrast, buccal mucosa offers a smooth and relatively immobile surface for long lasting controlled release systems (2, 10).

Before reaching vasculature, drugs have to be diffused through different layers in oral mucosa such as hydrophilic mucus, keratinized layers if applicable, densely packed epithelial cell layers, basement membrane and hydrophilic connective tissue. Any of these layers may pose as a major barrier in transport of drugs based on their properties such as molecular weight, lipophilicity, partitioning and solubility. It was generally observed that the top epithelial layer of thickness 200 µm is a major rate limiting factor in transport kinetics of drugs and proteins (9, 12). This permeability barrier is attributed to densely packed cells and so-called membrane-coated granules (MCG). MCGs are spherical/oval granules of 1-3 µm in diameter. These are located in intercellular spaces of intermediate cell layers at the top third of both keratinized and non-keratinized epithelium. The partitioning coefficient and solubility of drugs play more significant role than molecular size in their transportation across epithelium layer. Increased lipophilicity helps in the permeation of drugs through cell membranes and epithelium. (9).

The two major pathways of drug diffusion in the oral cavity are: a) transcellular, where compounds transverse across cells and b) paracellular, where compounds diffuse through intercellular spaces. The preferred route of compounds depend on their physiochemical properties. Because intercellular spaces are hydrophilic, and cell membranes are lipophilic in nature, they act as barriers to lipophilic and hydrophilic drugs respectively. Several absorption enhancers are being investigated and used to improve diffusion of molecules (2, 9).

2.3 Mucoadhesive Drug Delivery Systems

Mucoadhesive systems are composed of water soluble polymers that become hydrated up on contact with mucous tissue. These hydrated polymers undergo contact mixing with the mucin layer of mucous tissue to some depth, and form semi-permanent adhesive bonds, entanglements, and secondary chemical bonds. Bioavailability of drugs is increased by mucoadhesive systems because of their prolonged residence time at the application site. Intimate contact between a mucoadhesive and absorbing tissue provides high flux of drugs. Mucoadhesives can also increase the permeability of high molecular weight molecules, such as proteins (4).

2.3.1 Mechanism of action

The adhesion of mucoadhesives to tissue is the prominent feature of system, by providing several unique properties and advantages as described in former section. Various theories have been proposed in literature explaining possible mechanisms of the mucoadhesion (3, 4). Mucin, which coats epithelium, plays dominant role in the adhesion of mucoadhesives. Polymers interact with the mucin and adhere in one or more of the following ways:

i) Electronic theory: Adhesion of polymers to mucin is formed due to their electronic interactions with the glycoproteins. Electrostatic attraction between positively charged polymers and negatively charged mucin generally favors the adhesion.

ii) Adsorption theory: Chemical interactions between polymers and mucin develops adhesion. Primary chemical bonds or secondary interactions such as van der Waals forces, hydrogen bonds and hydrophobic bonds are responsible for the adhesion.

iii) Wetting theory: The ability of polymers in liquid formulations to spread over mucin determines adhesion of a system.

iv) Diffusion theory: The mutual diffusion of mucin glycoproteins and polymers to form interpenetrable layer and entanglements is responsible for the adhesion. Factors such as molecular weight, hydrodynamic size affects diffusion of polymers in to mucin.

v) Mechanical theory: Surface roughness on delivery systems determines the adhesion and increased roughness favors the adhesion due to increased contact area.

2.3.2 Factors affecting mucoadhesion

Mucoadhesion is affected by several physiochemical properties of polymers such as molecular weight, concentration, charge, functional groups, and the environment. While low molecular weight polymers can penetrate easily into mucus, high molecular weight polymers can have more entanglements with mucin. Hence ideal molecular weight is desired to achieve balance in diffusion of chains and their entanglements. The minimum molecular weight of polymers being used in mucoadhesive systems is desired to be 100,000 Da (10). However, ideal molecular weight is unique to each type of polymer, because of other properties such as flexibility, charge, and secondary interactions. Minimum amount of polymers also known as critical concentration, is required to achieve a stable interaction with mucus. While concentrations above the critical concentration decrease the flexibility and diffusion of polymers, low concentrations result in less polymer interactions per unit area of mucus, than required for stable adhesion.

Owing to the negative charge of mucin, ionic polymers, specifically cationic polymers, may exhibit better adhesive properties. Presence of functional groups on the polymers to form secondary bonds improve adhesive properties of the systems. Rate of hydration and swelling determines residence time of the systems and release profiles of active molecules. Environmental factors such as pH of saliva changes the ionization state of polymers, and may cause different behavior of the systems (4, 10).

2.4 Mucoadhesive Polymers

Several polymers are being investigated and used in mucoadhesive systems by different groups. These polymers can be classified based on different characteristics such as their source, charge, solubility and new generation of specific polymers.

2.4.1 Natural polymers

These polymers are synthesized naturally, and extracted from organisms and plants. Some examples of these polymers are agarose, gelatin, hyaluronic acid, and chitosan (10). Chitosan exhibited excellent mucoadhesive properties because of its ability to form strong electrostatic interactions with mucin, and supported by hydrogen bonds (3). Presence of functional groups in chitosan, such as amines and hydroxyls also provided opportunities to crosslink and derivatize new products. Several other natural polymers such as xanthan, gellan, pectin, sodium alginate, guar, and hakea were also used in composing mucoadhesives (10).

2.4.2 Synthetic polymers

Synthetic polymers are usually designed to resemble structures of natural polymers, but with slight modifications to enable the enhancement of desired properties. These polymers possessing high molecular weight, new functional groups, and charged groups help in forming controlled three dimensional network (10). Some of the widely used synthetic polymers in the mucoadhesives are cellulose derivatives, acrylic acid polymers, and vinyl polymers (4).

Cellulose derivative group of polymers such as carboxymethyl cellulose, hydroxypropyl cellulose, hydroxyethyl cellulose, and methyl cellulose possess cellulose backbone with modified hydroxyl groups and carboxyl groups. Presence of the carboxyl groups attracts water and cause enormous swelling and diffusion of the polymer chains. These carboxylic groups also deprotonate and form ionic interactions, andhydrogen bonds with mucin oligosaccharides. Polymers based on acrylic acid and their derivatives includes carbopol, poycarbophil, poly(methacrylate), and poly(ethylene glycol). In addition to their hydration properties and hydrogen bonds, they exhibit ability to interact with the thiol groups of mucin. Vinyl polymers such as polyvinylpyrrolidone and polyvinyl alcohol possess good hydrogen bonding capacity to mucin in addition to their swelling properties.(3, 4, 10).

2.4.3 Polymers with specific interactions

New generation of polymers are being developed to achieve specific interactions with mucin and cell surfaces. One such type of polymers, known as thiolated polymers, are made by modifying one of the mucoadhesive polymers with side chains possessing additional thiol functional groups. Thiol functional groups form covalent bonds (disulfide bridges) with the cysteine rich subdomains of the mucin glycoproteins. Formation of these primary bonds improved mucoadhesion greatly compared to their original polymers. Some examples of these polymers are poly(acrylic acid)/cysteine, chitosan/N-acetylcysteine, alginate/cysteine, chitosan/thio-glycolic acid, chitosan/thioethylamidine (3).

Lectin-mediated bioadhesive polymers, another kind of specific polymer, are made by conjugating lectin to the original mucoadhesive polymers. These polymers are capable of attaching specifically to the epithelial cell surface in contrary to nonspecific interactions to the mucin. Such direct adhesion of the polymers to a cell surface increase residence time, because the polymers cannot be washed away even though mucus and saliva are continuously being washed away and replaced by fresh mucus and saliva in short times. Lectins, found in bacteria and plants, achieve specific interaction by binding to certain sugars on the cell membrane. Other molecules capable of specific interactions, such as fimbriae (key role in bacterial adhesion) and antigens are also being conjugated to traditional mucoadhesive polymers to further improve residence times of mucoadhesives systems(10).

2.4.4 Polymer blends

Blends of structural and adhesive polymers are also being developed to obtain mucoadhesivity and strong patches simultaneously (4). For buccal absorption of different drugs, mucoadhesive films were prepared from blends of: chitosan and copolymer of poly vinyl alcohol and polyethylene; chitosan, polyvinylpyrrolidone, and gelatin; copolymer of methylvinyl ether and maleic anhydride; and polyvinylpyrrolidone plus carboxymethyl cellulose (13-16). Each system, however, has limitations such as burst release, complexity of preparation, and compatibility with the drug. Consequently, research continues to identify polymer blends with desirable properties.

2.5 Local Oral Disorders

Many of the approved mucoadhesive films/tablets such as BEMA technology (Bio Delivery Sciences International, Raleigh, NC), Striant^{TM}, Nitrogard^{R}, Fortfivo XL^{R}, are only being used to deliver drugs systemically. These systemic drugs are targeted to treat different disorders such as pain relief (cancer treatment, heart disease), antidepressants and supplementation of hormones (testosterone). Although some films are being investigated for treatment of local disorders, none of them are not commercially available. Some of the local oral disorders which will be addressed in this research work are discussed below.

2.5.1 Oral squamous cell carcinoma

Oral squamous cell carcinoma (OSCC) refers to any malignant cancer that arises from squamous epithelial cells in the oral cavity. All tumors are result of multistep process of accumulated genetic alterations (17). In this multistep process of progression toward OSCC, precancerous lesions characterized by hyperplasia and dysplasia are frequently observed (17). While hyperplasia refers to abnormal proliferation of cells, which can be controlled again with normal regulatory mechanisms, dysplasia refers to abnormality in maturation of cells (18). Dysplasia can be characterized by loss of normal epithelial stratification within the oral tissue, loss of polarity in the epithelial cells, nuclear pleomorphism and hyperchromasia, abnormal single cell keratinization (dyskeratosis), and increased or abnormal mitoses (19). Dysplasia is graded as mild to severe based on the accumulated nuclear abnormalities. As the dysplasia becomes severe, these abnormalities become more marked at increasing depth into the epithelium. The chances of developing carcinoma depend on the severity of dysplasia. Squamous cell carcinoma develops from dysplastic oral mucosal lesions if an early treatment has not been made (19). Hence treatment of the cancer at the dysplastic stage has enormous potential for decreasing incidence, metastasis, and improving survival periods (20).

Leukoplakia and erythroplakia are clinically applied terms in identifying the oral precancerous lesions. According to the WHO, leukoplakia is a clinical white patch or plaque that cannot be characterized clinically or pathologically as any disease. Leukoplakia lesions results from chronic irritation of mucous membrane by carcinogens, which stimulates the proliferation of white epithelial cells and connective tissue (21). It is the most common form of oral precancer, representing 85% of oral premalignancy (19). The potential of leukoplakia to convert to malignant cancer can only be confirmed on biopsy, based on association with dysplasia; if not associated with dysplasia, there is <5% chance of converting to malignant cancer (18).

Erythroplakia is generally characterized by superficial, friable red patches adjacent to normal mucosa. Similar to leukoplakia, it refers to a red patch that cannot be characterized clinically or pathologically as disease. This form is associated with epithelial dysplasia ranging from moderate to severe dysplasia or carcinoma. Several studies indicate that 40% of erythroplakia forms have converted to malignant cancer (18, 21).

2.5.1.1 Treatment options

The type of treatment for OSCC depends on the stage and location of the tumor. The best available treatments are surgery, radiation therapy, or combination of the two. These treatments are preceded and followed up by chemotherapy to activate cancerous cells to radiation and to avoid metastasis respectively.

Surgery is the mainstay treatment of the early stage oral squamous cell carcinomas (22). Primary tumors with adequate margins are carefully resected using craniofacial approaches involving osteotomies of mandible and selective neck dissections to access internal parts of oral cavity (22). Morbidity and postoperative disabilities resulting from surgery are the major disadvantages of this treatment approach.

Radiation therapy can be a good alternative to preserve function, cosmesis, and to avoid the morbidity associated with a major operation. It is also used in combination with surgery postoperatively to remove the residual cancer and preoperatively to render an advanced unresectable cancer to complete surgical removal (23). Even though this kind of treatment can be successful, unavoidable exposure of the operator to radiation, and nursing care required for the duration of treatment are some of the drawbacks. Radioimmunotherapy, a recent development that involves the use of radiolabeled monoclonal antibodies specific to cancer cells. Lack of monoclonal antibodies with a high specificity for head and neck cancers, however, is slowing down the advancement of this technique. Promising results have not been achieved for solid tumors, but this approach is being considered as a good alternative treatment (24, 25)

Photodynamic therapy, one of the newer alternatives to radiation therapy and surgery (26), involves use of a photosensitive prodrug that is injected into the body by systemic delivery. After the prodrug reaches the tumor, it is activated by exposure of tumor to laser light of specific wavelength. Many groups are evaluating this therapy for treatment of squamous cell carcinoma, especially for early stages of oral and neck cancers (27). However, huge loss of drug due to systemic delivery, uncertain long-term results, and various other factors, such as drug dosage, time interval between dye administration and light application, and avoiding sun for a minimum of 30 days, are some of the drawbacks. Hence photodymanic therapy has not yet been used widely in treatment of oral cancers (26, 27).

2.5.2 Oral mucosal wounds and their regeneration

Wound healing is a complex process involving multiple cells, cytokines, and growth factors which occurs in different phases known as hemostasis, inflammation, proliferation and remodeling. Although these phases occur separately, they widely overlap in time, molecules, and space (28). Any change in proper sequence, specific time, and duration of biophysical functions results in impaired healing and fibrosis.

Oral mucosal wounds are observed to heal faster at an average time of 2 weeks with no or minimal scar formation. This was observed and reported in several rodents, big animal studies and human clinical cases (28, 29). Faster and scarless healing of the oral mucosa is attributed to several reasons such as higher reepithelialization rate, faster proliferation of fibroblasts, small amount of immune mediators, and ready availability of multipotent stem cells. In addition presence of saliva is believed to decrease immune response, fibrosis, and provides several growth factors (29).

Although the oral mucosa exhibits faster healing, these processes may not help in large wounds such critical size defects and/or where chunks of tissue are lost. Such injuries are generally observed in trauma patients, battlefield injuries and surgical wounds. Craniomaxillofacial injuries account for 15-34% of general trauma and 26% of battlefield injuries (30, 31). Oral mucosal deficiencies are created in several clinical conditions, such as post-neoplastic ablation, periodontal pathologies, tooth replacements, and preparation of oral mucosal grafts for urethral reconstructive surgery (32, 33).

Treatment options of these defects include use of autografts extracted from other mucosal surfaces. This approach however results in morbidity due to second surgery and also limited by available mucosal tissue for harvesting. Owing to advances in tissue engineering and decellularization process, use of allografts and lab grown tissue engineered oral mucosal grafts showed short term clinical success (34, 35). These *ex vivo* mucosal grafts, however, are also limited by some factors such as reduced viability, difficult handling, and fabrication time of 4-6 weeks (29).

2.5.3 Other major disorders (ulcers)

Oral mucositis (OM), an inflammatory ulcerous oral wound condition, is a commonly occurring side-effect of anti-cancer therapies, including chemotherapy and/or radiotherapy (36). The incidence rate of the OM is 30-75% in patients subjected to chemotherapy and 70-90% of bone marrow recipients (36-38). It was found even more for head and neck radiotherapy, exceeding 90% (39, 40). This painful and debilitating condition is manifested by erythema and inflammatory lesions, which rupture through the oral epithelial mucosal walls, compromising the patient's overall quality of life by affecting routine functions, such as eating, swallowing, and speaking.

Current treatment options of this disorder address more on symptom management rather than halting of damage and regeneration of damaged tissue. Oral mouth rinses (Caphosol™) give rapid relief due to their moisturizing effect but do not aid healing. Although some oral rinses, such as "Magic Mouth Wash", have combinations of ingredients, including antibiotic, antihistamine, antifungal, steroid, local anesthetic, and/or antacid, they are ineffective in treating OM and had only therapeutic value equivalent to a non-therapeutic saline mouthwash. (37, 41, 42).

One possible reason for failure of oral rinse products may be due to lack of drug localization because of short residence time. The oral cavity is a complex environment that experiences continual salivary flushing, which helps to provide a hydrating medium for drug distribution, but such effects also result in rapid drug clearance by swallowing. In overcoming such drawbacks, a drug delivery device should be capable of minimizing salivary dilution effects, thereby potentially reducing/obviating the need for repeated drug dosing. Bioadhesive gels, such as Gelclair and Zilactin, are an alternative to washes.

Although these gels can be applied to irregular surfaces and can have increased residence time compared to mouth rinses, they may still remain susceptible to shear forces from the tongue and cheeks and continuous salivary flushing. The presence of a non-degradable and hydrophobic backing layer in the proposed mucoadhesive film in this dissertation can protect mucoadhesive components from all these forces.

2.6 Specific Aims

This dissertation research was guided by the following specific aims.

- Develop a mucoadhesive system loaded with imiquimod by addressing loading and solubility problems due to imiquimod hydrophobic nature.
- Characterize adhesive, mechanical properties, swelling, and release properties and compare them with change in composition of polymers.
- Analyze permeability and transport kinetics of films *ex vivo* and check their bioactivity *in vitro.*
- Identify residence time of films *in vivo* in humans and hamsters followed by quantification and distribution of the drug in the local mucosal tissue and blood of hamsters.
- Develop epidermal growth factor (EGF) loaded films and investigate their bioactivity, and efficacy on wound healing of oral mucosal tissues *in vitro*.

Chapter 3 Development of Imiquimod-Loaded Mucoadhesive Films for Oral Dysplasia

3.1 Introduction

Oral cancer is the eight and eleventh most common human neoplasm in men and women, respectively, and is expected to account for 2.5% of all newly diagnosed cancers in 2012 (21). Nearly 40,250 new cases and 7,850 deaths are expected in the United States alone in 2012 (21). Low cancer survival rates can be improved by early diagnosis, because survival is directly related to the stage of the disease at the time of diagnosis (20). Treatment at an early, precancerous stage is the most desirable management strategy, avoiding field cancerization, metastasis (20), and progression of disease. Precancerous dysplastic lesions of the mouth and skin have an advantage in detectability because they are externally visible. For oral lesions, however, current treatments, such as surgical resection, radiation, and chemotherapy, are primarily administered after the disease has already progressed to oral squamous cell carcinoma (OSCC). Furthermore, resection of dysplastic regions can lead to postoperative disabilities. Hence, unlike standard invasive treatment approaches, this study was designed to develop a noninvasive and local treatment approach.

Oral dysplasia refers to premalignant changes preceding OSCC. The chances that carcinoma will occur depend on the severity of dysplasia. Several compounds, such as vitamin A, carotene, retinoids, antioxidants, lycopene, fenretinide, and genistein, are being investigated for their potential to stop the progression of oral dysplasia to OSCC (14, 43-45). However, imiquimod, the only immune response modifier approved for market (as Aldara), has been successfully used to treat actinic keratosis and superficial basal cell carcinoma (sBCC) (7, 46, 47). In addition, the off-label use of imiquimod cream has been successful in treating precancerous lesions and malignancies of several mucosal surfaces, including neoplasm of the vulvar epithelium, lip ulcers, erythroplasia of Queyrat, melanoma of the intraepithelial oral mucosa, and even oral leukoplakia (8, 47-50). However, all of these procedures used the original (Aldara) cream to treat lesions in the oral cavity. Creams are typically not used intraorally because they can be easily washed away with continuous saliva turnover and movements of the mouth. This washing can cause the drug to affect the surrounding healthy tissue and may result in systemic side effects because of intestinal absorption. These deficiencies highlight the need for an imiquimod delivery system that increases residence time on the mucosal surface, enhances bioavailability, and targets specific lesion sites.

Mucoadhesive drug delivery systems are being used to improve the efficiency of drug delivery (4). The dual ability of these systems to adhere to mucosal surfaces and to control drug release may provide a better system for imiquimod delivery than Aldara cream. Intimate contact between mucoadhesive and absorbing tissue provides a high flux of drug and increases bioavailability (4). These properties of mucoadhesive drug delivery systems provide the advantage of localizing the drug on mucosal surfaces, thereby avoiding first-pass metabolism and the adverse effects associated with systemic drug delivery. The versatility of mucoadhesive systems also allows easy use of various drugs and proteins with only slight changes in the processing of films.

Aldara cream contains imiquimod in a hydrophobic formulation containing fatty acids. However, in an effective mucoadhesive system the drug must be uniformly dispersed in hydrophilic polymer solutions and released in a hydrophilic environment (mucus). The lack of knowledge about the use of imiquimod in hydrophilic systems required the exploration of various methods of loading the drug delivery system and the subsequent release profiles.

Commercially available mucoadhesive systems, such as BEMA technology (Bio Delivery Sciences International, Raleigh, NC) and most others (4), were designed to last for 15 to 45 minutes and to provide systemic drug delivery. In contrast, an effective treatment approach for precancerous oral lesions requires local delivery and longer residence times of the drug on only the buccal mucosa. The study reported here used bioerodible and FDA-approved polymers, polyvinylpyrrolidone as a structural polymer and carboxymethylcellulose as an adhesive polymer, to provide a flexible polymer matrix for the control of drug release.

The overall objective of this study was to design and characterize a bilayered mucoadhesive drug delivery film for localized delivery of imiquimod. Because of the highly hydrophobic nature of imiquimod and the challenges associated with improving its solubility in hydrophilic polymers, the present study also sought to evaluate various methods of increasing drug loading in the films and to evaluate the subsequent effects on *in vitro* release of bioactive drug.

3.2 Materials and Methods

3.2.1 Chemicals

Pure imiquimod was purchased from CalBiochem (White House Station, NJ). Polymers used for making films were polyvinylpyrrolidone (PVP) K-90 (Spectrum; New Brunswick, NJ) and carboxymethylcellulose (CMC; sodium salt, medium viscosity; Sigma, St. Louis, MO). Other chemicals used were propylene glycol, ethanol (190 proof), 2-hydroxypropyl-β-cyclodextrin (HPβCD; cell culture tested), poly(ethylene-co-vinyl acetate) (PEVA; 18wt% vinyl acetate), (Sigma-Aldrich, St. Louis, MO); DMEM/high glucose with 10% FBS (Hyclone Laboratories; South Logan, UT). Tumor necrosis factor (TNF)-α was measured using a commercial ELISA kit (eBioscience; San Diego, CA).

3.2.2 Fabrication of films

A 40% w/v aqueous solution of structural polymer PVP was mixed with ethanol at 1:1 v/v (PVP solution to ethanol), followed by the addition of 50% v/v propylene glycol as a plasticizer. Concurrently, a 2% w/v aqueous solution of mucoadhesive polymer CMC was prepared. Pure drug or drug solution was added to the combined PVP and CMC polymer solutions, thoroughly mixed using heavy duty rotator at high speed (Roto Torque, Cole Parmer, Chicago, IL), and left overnight at 43°C to remove bubbles. The polymer solutions were cast in Teflon dishes 50 cm^2 in area and dried at 60ºC for 8 hours, and stored in desiccators until used. Mass and thickness of samples (diameter, 1 cm unless otherwise noted) punched from random points ($n \geq 10$) of films were measured to ensure uniformity.

3.2.3 Methods of loading imiquimod

Imiquimod was loaded into polymer solutions by four methods. The first method used sonication (ultrasonic processor at an amplitude of 30 W) to disperse the imiquimod in the CMC solution before it was combined with the PVP solution. The second method solubilized imiquimod in linoleic acid (10 mg/mL) and mixed it with CMC solution and then with PVP solution. The third method used HPβCD to form complexes with imiquimod by co-evaporation (51). In brief, separate solutions of aqueous HPβCD and drug dissolved in methanol (0.46 mg/mL) were mixed at a 1:1 molar ratio and shaken (150 rpm) at 43°C for 24 hours. Half of the methanol was evaporated from this solution until near-saturation (before complexes precipitated) and poured into the PVP-CMC polymer solution. The remaining methanol was evaporated by drying at 60°C. The fourth method dissolved imiquimod in 3:7 v/v acetate buffer (pH 4.0, 100 mM):methanol and mixed it with the polymer solution. The amount of drug loaded in all films was 18 mg, unless otherwise noted.

3.2.4 Characterization of HPβCD-imiquimod complexes

3.2.4.1 Phase solubility

Phase solubility studies (52) were conducted to determine the association constant and favorable molar ratio for complexes formed between HPβCD and imiquimod. An excess amount of drug was added to increasing molar concentrations of completely dissolved aqueous solutions of HPβCD and was incubated at 50°C with shaking at 180 rpm for 100 hours. Undissolved drug particles were filtered (0.45 µm) from the solutions, and the filtrate solutions were analyzed by UV absorbance at 244 nm. The shape of the graphed relationship between concentration of a guest molecule and HPβCD (Figure 3.1) was used to determine the apparent molar ratio of imiquimod and HPβCD complex formation.

The association constant of the complex (1:1) was then calculated using the following formula

$$k_{1:1} = \frac{slope}{s(1-slope)}, \qquad \text{[Eq. 1]}$$

where s represents intrinsic solubility (solubility of imiquimod without cyclodextrin = 0.1 mM; (52). The aqueous solubility of these mixtures was used as a criterion for selecting the best method of forming complexes.

3.2.4.2 Differential scanning calorimetry (DSC)

Formation of HPβCD-imiquimod complexes was confirmed by DSC analysis. Samples (1-3 mg) of pure imiquimod, lyophilized HPβCD, physical mixture of lyophilized HPβCD and imiquimod, and lyophilized HPβCD-imiquimod complex (1:1) were heated from 30°C to 300°C at a rate of 10°C per min. Samples were hermetically sealed in pin-holed aluminum containers and ran with an empty aluminum container as blank in a differential scanning calorimeter (DSC Q 200, TA Instruments, Newcastle, DE). The environment was maintained inert with the presence of nitrogen gas at flow rate of 50 mL/min.

3.2.5 pH of films formulated with acetate

Because residual acid from the formulations with acetate buffer could irritate and damage normal mucosal cells, the pH of the mucoadhesive films and the release supernatants was measured. The surface pH of the films was determined by incubating samples 1.4 cm in diameter on 2% (w/v) agar plates. Because mucoadhesive films eroded, their surface pH was measured with pH strips at predetermined intervals up to 12 hours. For release supernatants, samples were dissolved in 1 mL of simulated saliva (SS; 16 mM Na_2HPO_4, 1.3 mM KH_2PO_4, 136.9 mM NaCl, pH=6.75; (53). Solution pH was measured by both a pH meter and pH strips after 6 hours, when the films had been completely dissolved.

3.2.6 Release studies

Samples punched from random points of cast films were attached to the wall of 6-mL polyethylene vials so that the release of drug could be limited to only one side. These samples were immersed in 6 ml of SS and incubated at 37°C with shaking at 150 rpm. Supernatants were collected and stored at predetermined intervals and were then replaced with fresh SS. Concentrations of imiquimod released into the supernatants were measured by using fluorescence at an excitation wavelength of 250 nm and an emission wavelength of 340 nm. Samples of all film types had equal drug loading (280 µg) to ensure uniform comparison of their release profiles.

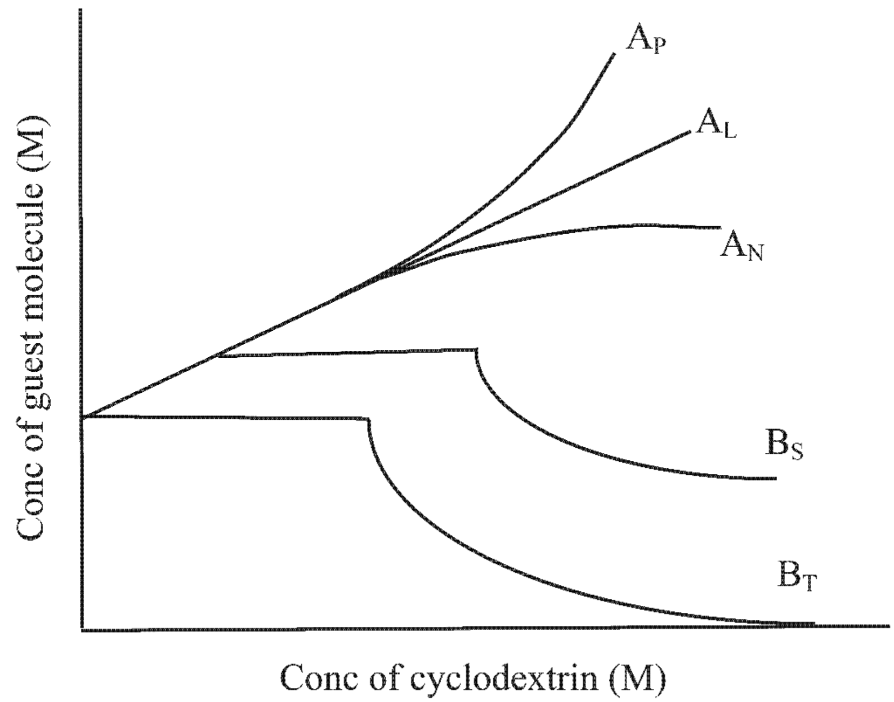


Figure 3.1 Phase solubility diagrams corresponding to various types of complex formation. A_p, guest molecule binding to cyclodextrin with molar ratio greater than 1; A_L, guest molecule binding to cyclodextrin at 1:1 molar ratio; A_N, not clearly understood; B, dissociation after reaching saturation. Adapted from (52).

3.2.7 Effect of residual water content of films on drug release

To assess the effect of alterations in residual water content on drug release, studies were conducted on films obtained at various stages of drying. Samples (HPβCD-imiquimod formulations were punched during the process of drying at 6 hours (under-dried) and 8 hours (normal). Release studies were performed according to the procedure described previously. Residual water content of films was determined by drying the obtained films at 60°C to a constant mass for an additional 24 hours.

3.2.8 Backing layer

PEVA films were prepared by casting 10% w/v PEVA in toluene into Teflon dishes. The dishes were then dried at 30°C for 48 hours in sealed containers so that cracks in the films could be avoided by slowing the evaporation of the solvent. Bilayered films were subsequently prepared by casting mucoadhesive polymer solution onto freshly cast PEVA film and drying it at 60°C.

Bilayered film samples 1.5 cm in diameter were incubated in 5 mL of SS (37°C, 150 rpm). The interface of the mucoadhesive component and the PEVA backing was examined visually for up to 6 hours while being shaken in SS so that any detachment caused by time and erosion could be detected. Samples were also collected at regular intervals and examined qualitatively so that detachment between both films could be assessed.

The permeability of imiquimod through the PEVA backing layer was determined by using a Franz cell apparatus. Samples with a diameter slightly larger than that of the Franz cell were punched from a bilayered acetate formulation film. The sample was oriented so that the backing layer faced the receptor compartment and the mucoadhesive side faced the donor compartment. After SS was added to the receptor and donor compartments, the Franz cell was incubated at 37°C overnight with shaking (150 rpm). The release of imiquimod into the receptor compartment was analyzed after 24 hours.

3.2.9 *Ex vivo* mucoadhesion time and effect of thickness

Film samples (n=5) punched from drug-free bilayered films and Aldara cream of similar mass were attached/spread to/on the mucosal surface of pre-hydrated (50 µL SS) porcine buccal tissue. Tissue samples were attached to a glass slide with cyanoacrylate glue. The glass slide was fixed to the moving actuator of a BOSE ELF3300 mechanical testing system and allowed to move up and down into 700 mL of SS at a rate of 18 cycles per min. The patch was completely immersed in the buffer solution at the lowest point and was out of the solution at the highest point. The time at which the backing layer completely detached or cream was completely washed off from the tissue was recorded as the *ex vivo* mucoadhesion time. In addition, thicker (0.39 mm) films were also prepared and were compared with the thinner (normal; 0.30 mm) films so that the effect of thickness on mucoadhesion time could be assessed.

3.2.10 Bioactivity assay

Mucoadhesive films loaded with imiquimod by formulation with acetate buffer were selected for bioactivity testing. The bioactivity of imiquimod in release supernatants was assessed by determining the production of TNF-α by RAW 264.7 cells (TIB-71; ATCC, Manassas, VA) when exposed to imiquimod. RAW 264.7 cells were allowed as many as 5 passages before being used in this assay. Cells were suspended in DMEM/high glucose medium at 1×10^5 cells per mL and were seeded into 24-well plates. They were allowed to equilibrate for 20 hours before the addition of sterile-filtered samples (60µl). The resulting concentration of imiquimod in wells was 5 µg/mL. Cells were again incubated for 12 hours in a humidified incubator at 37°C and 5% CO_2. The concentration of secreted TNF-α was then measured with a commercial ELISA kit.

3.2.11 Statistical analysis

All experiments were conducted in triplicates and were repeated at least once so that the reproducibility of results could be demonstrated. The results were expressed as means ± standard error of the mean. Although unpaired two-tailed Student *t*-tests were used to compare the instantaneous release of drug by dried and normal films, ANOVA with the Tukey post hoc test was used to compare instantaneous drug release across the various formulations. Results were considered statistically significant at the level of $p<0.05$.

3.3 Results

3.3.1 Morphology and characteristics of mucoadhesive films

Translucent and flexible films were peeled from Teflon dishes, except for those prepared with linoleic acid (Figure 3.2). Films prepared with linoleic acid were tacky, shrunken, and nonuniform, and their color became slightly yellow during the process of drying at 60°C. Films formulated with acetate buffer were also more adherent than the sonication and HPβCD films. Increased drying time resulted in brittleness and shrinkage of all types of films.

The addition of acetate buffer (pH=4.0) did not render either the films or the release supernatants acidic (data not shown). The surface pH of films on the agar surface and the pH of release supernatants ranged from 6.8 to 7.0 during the entire process of bioerosion.

3.3.2 Characterization of HPβCD-imiquimod complexes

3.3.2.1 Phase solubility studies

The amount of imiquimod incorporated into complexes with HPβCD increased linearly with increasing concentrations of cyclodextrin. A plot of imiquimod concentration as a function of cyclodextrin concentration showed A_L type behavior (Figure 3.3), a finding suggesting a 1:1 interaction between HPβCD and imiquimod. The association constant of these complexes was 23.3 ± 1.8 M^{-1}, and the maximum solubility of the 1:1 complexes was 100 ± 5 µg/mL.

3.3.2.2 Differential scanning calorimetry

The DSC thermogram of imiquimod was typical of a crystalline anhydrous structure with a sharp melting endothermic peak at 299°C. The thermogram (Figure 3.4) of HPβCD showed a broad endothermic peak from 30°C to 130°C (54, 55). This characteristic peak of HPβCD was also observed in thermograms of physical mixture and complex. The thermogram of imiquimod-HPβCD complexes shows the complete absence of the characteristic endothermic drop at 299°C suggesting the formation of imiquimod- HPβCD inclusion complex. However, the characteristic endothermic peak of imiquimod was shifted towards lower temperature 263°C with reduced peak intensity in the thermogram of physical mixture as observed in other studies (55-58).

3.3.3 Release of imiquimod

Drug release from films prepared with linoleic acid was not studied because of their non-uniformity. Sustained release was achieved with films prepared with the other three formulations (Figure 3.5a). All films demonstrated a small burst at 40 min followed by a sustained release for as long as 2 hours. After a small increase at 140 to 160 minutes, the release continually decreased thereafter. All films began disappearing from the vial walls at 140 to 180 minutes. The amount of drug released from acetate formulation at 20, 40, and

60 min was significantly higher than the amount released from sonication and cyclodextrin formulations ($p<0.05$). There was no significant difference in drug release between the sonication and cyclodextrin formulations. Although the amounts of drug released differed, the general pattern of drug release was similar.

The cumulative release of imiquimod showed that although 68% of drug was released from acetate formulation films, only 43% was released from the sonication formulation films and only 38% of drug was released from the cyclodextrin formulation films (Figure 3.5b). Cumulative release profiles of all films were linear; the coefficient of variation (r^2) ranged from 0.96 to 0.99, a finding strongly suggesting zero-order kinetics. Release data were also further analyzed according to the Korsmeyer-Peppas equation (45). The value of n was 0.89 for the acetate formulation, 1.37 for the cyclodextrin formulation, and 1.66 for the sonication formulation, a finding suggesting drug release by Super-Case II relaxation.

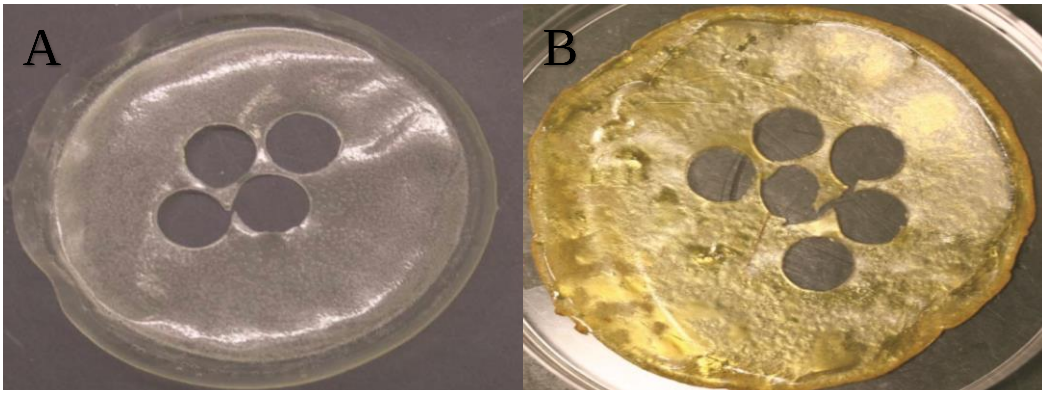


Figure 3.2. Representative appearance of cast PVP-CMC films. Acetate buffer, sonication, and HPβCD formulations were translucent (A), whereas films containing linoleic acid turned yellow and became nonuniform upon drying (B).

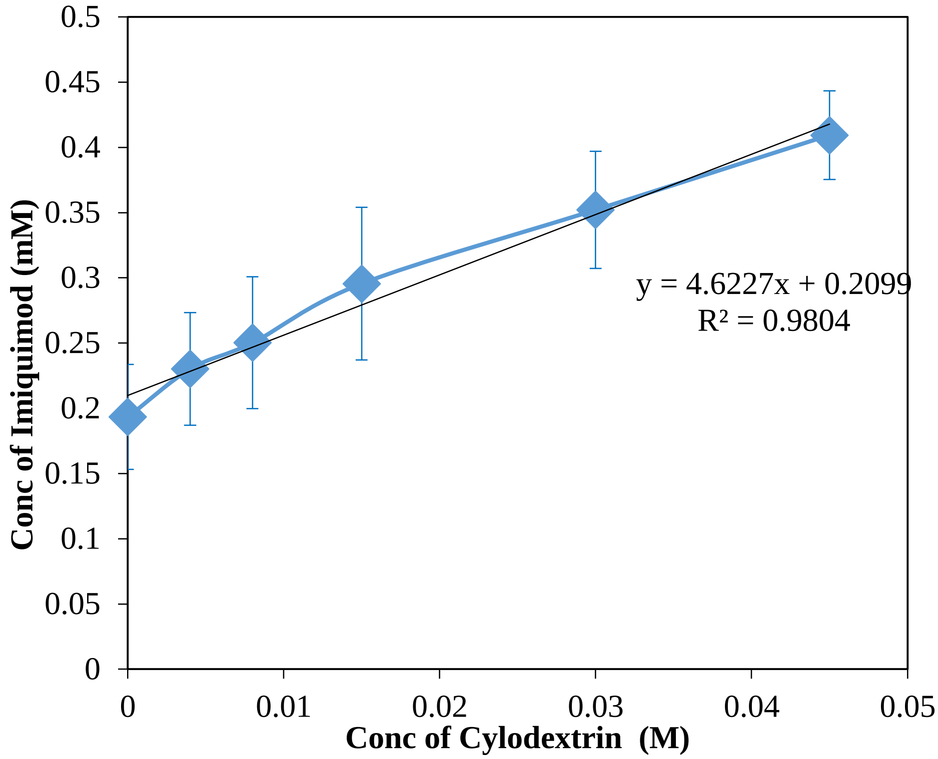


Figure 3.3. Phase solubility studies of 1:1 HPβCD-imiquimod complexes showing concentration of imiquimod incorporated into complexes as a function of cyclodextrin concentration. Data are shown as means ± standard error (n≥3).

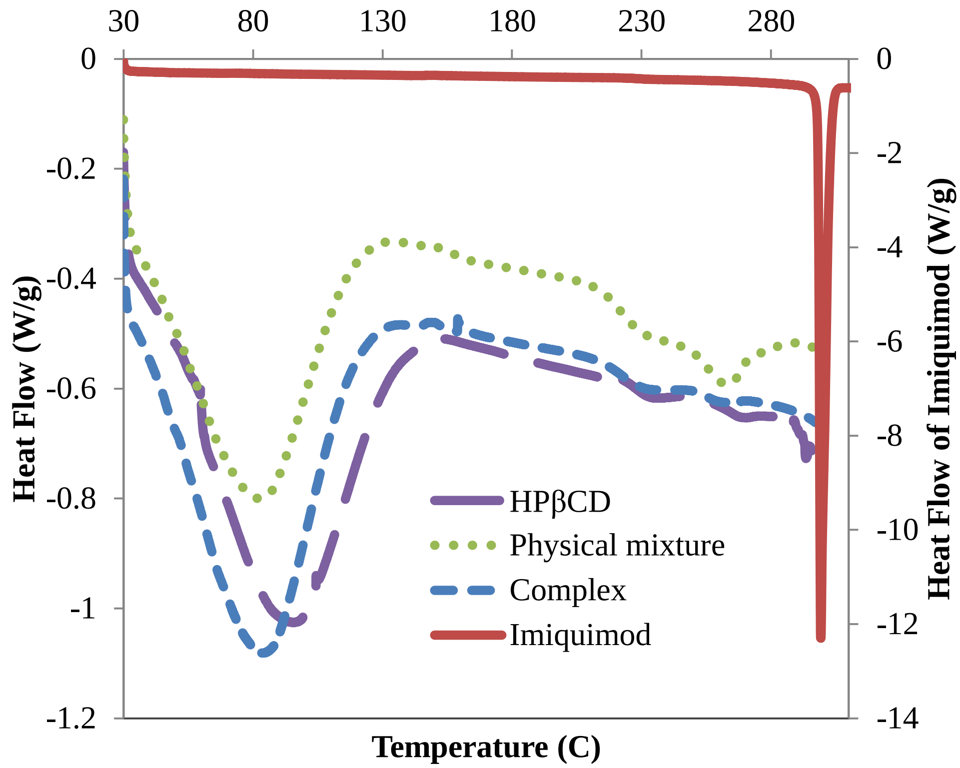


Figure 3.4. DSC thermograms of pure imiquimod, cyclodextrin, physical mixture of imiquimod and cyclodextrin, and imiquimod-cyclodextrin complexes.

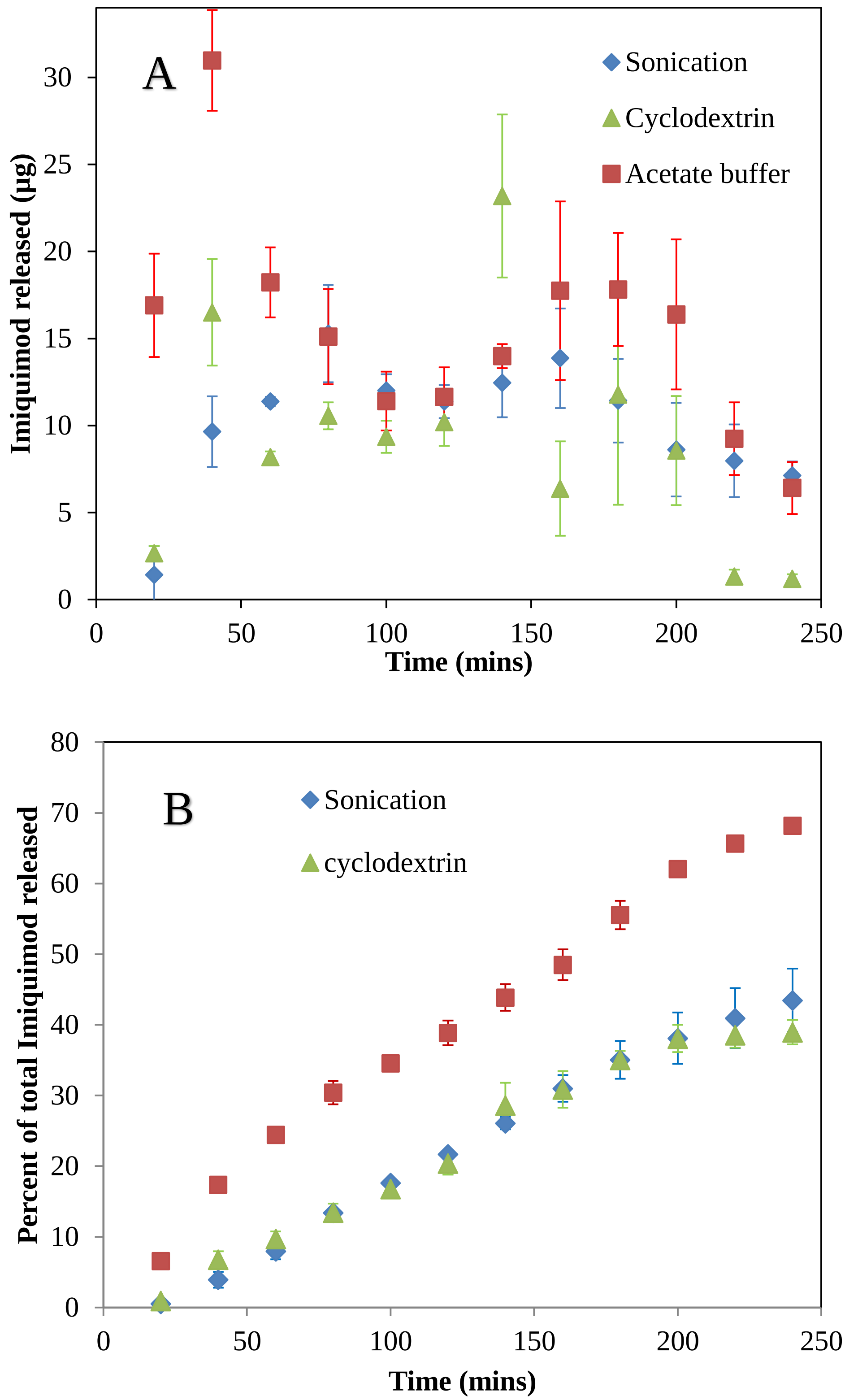


Figure 3.5. A) Instantaneous and B) cumulative imiquimod release profiles for films loaded with different methods. Data are shown as means ± standard error (n≥3).

3.3.4 Drug distribution

Films had an average mass of 21.6±1.69 mg and an average thickness of 0.23±0.020 mm, regardless of differences in loading and formulation. The average amount of drug released from samples randomly punched from the cast films was 139±21 µg for sonication formulations, 113±8 µg for cyclodextrin formulations, and 191±16 µg for acetate buffer formulations; the differences in these amounts were statistically significant ($p<0.0027$). Although sonication formulations exhibited a coefficient of variation (CV) of 15.7%, the drug was more uniformly dispersed in the other two formulations (CV of cyclodextrin formulations, 7.7%; CV of acetate buffer formulations, 8.5%).

3.3.5 Effect of residual water content of films on drug release

The residual moisture content of under-dried films (6 hours) was 52±1.13% compared to 32±2.13% in normal (8 hours) films. Film samples obtained after under-drying for 6 hours at 60°C eroded faster and did not provide sustained release for extended periods (Figure 3.6). They were also softer, tackier, and more delicate than normal films. Samples collected from these films exhibited an initial burst at 20 minutes followed by sustained release for 1 hour and the release of continually decreasing concentrations of drug thereafter. This finding was in contrast to findings related to normal films, which exhibited an initial burst at 40 minutes and maintained sustained release for 2 hours (Figure 3.6).

3.3.6 Backing layer

PEVA films 100 to 120 µm thick were formed. Thinner films 50 µm thick were also made by casting a smaller volume of PEVA-toluene solution, but the resulting films were too weak to withstand handling. No difficulty was encountered in peeling the bilayered films (Figure 3.7) and punching out samples. Visual examination and qualitative handling of these films when dissolved in simulated saliva showed that the backing layer was firmly attached to the mucoadhesive layer throughout the erosion process.

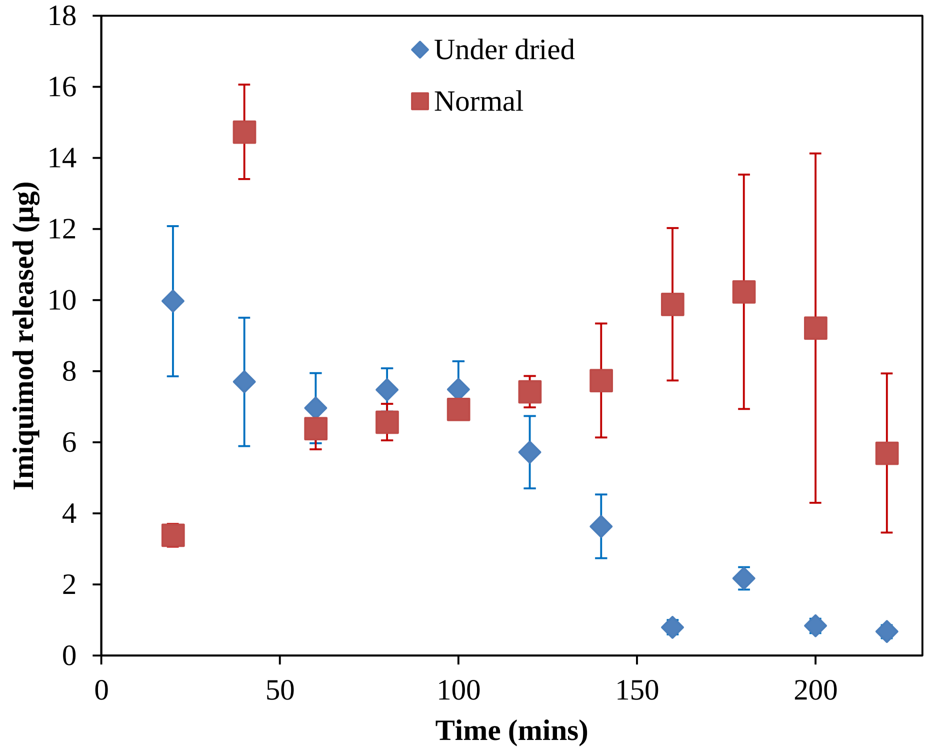


Figure 3.6. Instantaneous imiquimod release profiles of normal and under-dried films prepared with the cyclodextrin formulation. Data are shown as means ± standard error (n≥3).

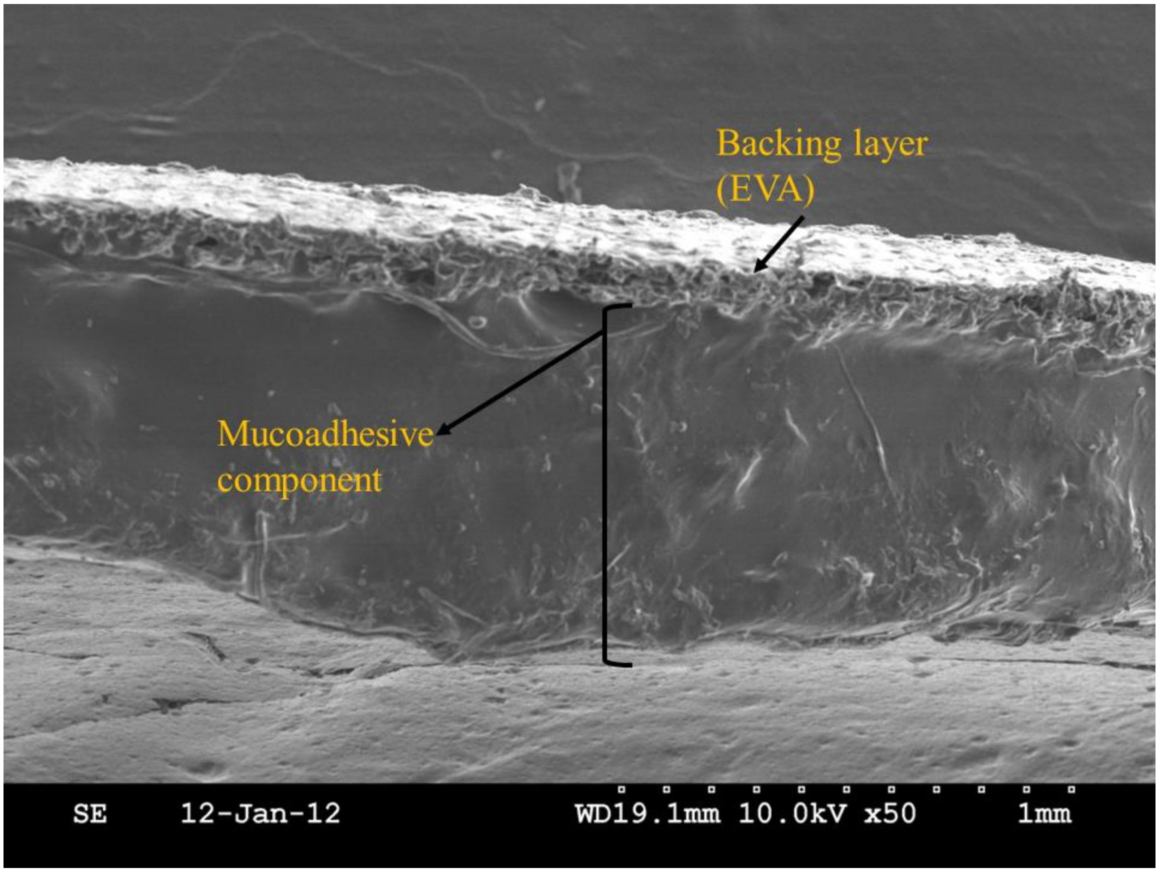


Figure 3.7. Representative scanning electron micrograph showing cross-section of a bilayered mucoadhesive film. The 900-µm-thick mucoadhesive component was backed by a 100-µm-thick PEVA layer.

The PEVA backing layer was impermeable to drug and was successfully used to limit the transport of drug to one direction. At 24 hours, the mucoadhesive component of bilayered films was completely dissolved into the simulated saliva present in the donor chamber of the Franz cell apparatus. However, the concentration of imiquimod in the receptor chamber was only 2% of the amount of drug found in the donor chamber, a finding suggesting the impermeability of the PEVA.

3.3.8 *Ex vivo* mucoadhesion time and effect of thickness

The average thicknesses of the two types of bilayered films tested were 0.30 and 0.39 mm. Both types of mucoadhesive films demonstrated their adhesiveness to buccal mucosa tissue. The *ex vivo* mucoadhesion time of the films was 10.45±1.8 for the thinner films and 5.95±0.7 hours for the thicker films; this difference was statistically significant ($p<0.01$). In contrast, 90% of Aldara cream was completely washed off within an hour showing the advantage of using mucoadhesive films.

3.3.9 Bioactivity assay

The amount (60µl) of sterile filtered supernatants added was equal to 12% of the medium. Hence as this exceeds normal 2% of foreign substance, toxicity of supernatants was analyzed with MTTS assay (results not shown) and no significant difference in activity of cells was observed. Imiquimod released into simulated saliva during film erosion stimulated the secretion of more than 1000 pg/mL of TNF-α from macrophagic (RAW 264.7) cells (Figure 3.8). There was no significant difference in the amounts of secreted TNF-α between the release supernatants from drug-loaded mucoadhesive films and pure drug solutions. No significant difference in the amounts of secreted TNF-α was observed between controls, blank solution, and blank films; this amount was less than 100 pg/mL.

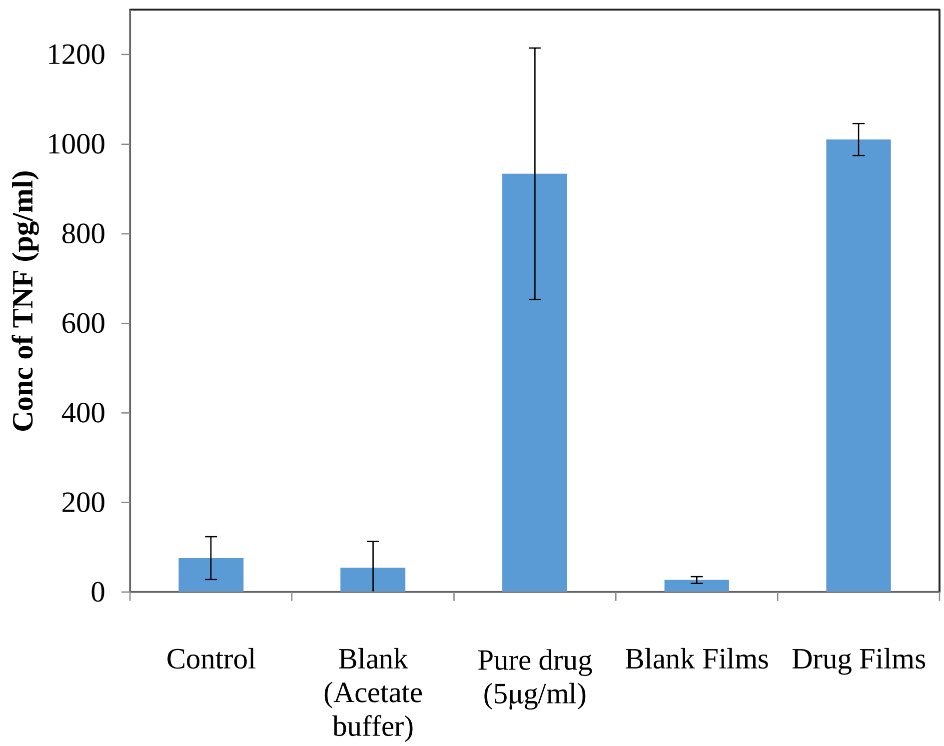


Figure 3.8. Amount of TNF-α produced by RAW 264.7 cells when induced by imiquimod either in pure drug form or in release supernatants released from film. Data are shown as means ± standard error (n≥3).

3.4 Discussion

Several drugs and biomolecules are being developed or investigated for use as chemotherapy for oral dysplasia (44). Compared with traditional systemic delivery, local delivery of such drugs can increase treatment effectiveness and avoid adverse effects. The versatility of mucoadhesive films designed in this work allows the use of nearly any biomolecule and could be used for several other applications in which localized modulation of oral cell and tissue responses is necessary. The system is equally adaptable to any mucosal surface, and, furthermore, the mechanical flexibility of films allows the treatment of nonuniform tissue defects, makes their application simpler, and enhances the release of bioactive agents to the intended site of action.

Several mucoadhesive drug delivery systems are being prepared and used for all types of mucosa, including gastrointestinal, vaginal, buccal, nasal, and ocular applications (4). Some of the most common polymers used in mucoadhesive systems are Carbopol® and its variants, sodium carboxymethylcellulose, hydroxypropylcarboxymethylcellulose, and polycarbophils (4). Although several mucoadhesive polymers exist, various groups have attempted to form blends of polymers that can improve film-forming properties and tensile properties and can alter drug release mechanisms and time of bioerosion (4, 59-61). A blend of PVP and NaCMC was selected for the current application (15). Although PVP was chosen for its film-forming ability, it also has adhesive power comparable to that of the mucoadhesive polymer NaCMC (53).

Off-label use of imiquimod in the treatment of dysplastic lesions involves the use of commercially available Aldara cream, applied 3 times per week for 8 to 12 weeks (8, 50, 62). Although overnight application of cream on the site may work well for skin cancer, the cream may not remain in the oral cavity for more than an hour. This problem can be overcome by the mucoadhesive films described here, which had an erosion time of 4 hours. The use of these films, therefore, can avoid the washing away of drug, increase residence time, enhance bioavailability, and provide better control of drug release.

The drug amounts chosen for loading into mucoadhesive films were based on the suggested dose of commercially available Aldara cream: 0.625mg/cm^2. However, the permeability of various drugs through the buccal mucosa is at least 4 to 4000 times more than the permeability through the skin (63). Although the required dosage for the buccal mucosa will ultimately be determined by animal and human trials, the development of the mucoadhesive film formulations described here was planned to enable the delivery of adjustable drug doses.

It is common practice to add drug directly to polymer solutions when drug-loaded films are formulated by the solvent casting method (13-15, 64). However, achieving uniformity in drug distribution when very hydrophobic drugs, such as imiquimod, are added to highly viscous hydrophilic polymer solutions can be challenging. Hence, this study evaluated various methods of loading imiquimod to suit various requirements, e.g., maximum loading and release kinetics. In a first attempt at achieving uniformity, polymer solutions were sonicated after the drug was added. Although this method of loading was easy to perform, the physical distribution of imiquimod into highly viscous PVP and CMC solution may be problematic when the process is scaled up. This finding is also supported by CV analysis of drug loading in the films: the films formulated by sonication had a CV of 15, which was twice that for the films formulated with cyclodextrin and acetate buffer. Linoleic acid, because of its high solubility for imiquimod (17 mg/mL; (65) and its similarity to the fatty acid oleic acid used in the original Aldara cream, was chosen as the next step for solubilizing imiquimod and mixing it with polymer solutions. The films formulated with linoleic acid were nonuniform, and the oxidation of linoleic acid during drying resulted in a rancid odor and possible degradation into byproducts. The production of these metabolites may interact with the drug and change its properties. In addition, oxidized linoleic acid metabolites activate TRPV1 channels and cause pain in rodents (66).

Cyclodextrins, cyclic oligosaccharides, have been widely used for the past 30 years as drug carriers that solubilize hydrophobic drugs (67, 68). The hydrophobic cavity surrounded by highly hydrophilic sugar molecules helps in the reversible binding of insoluble drugs and the formation of complexes (69). Although addition of di- or tri-block copolymers (such as the PEO-PPO-based Pluronics) could enhance dispersion of hydrophobic drugs, the number of polymeric components that could affect the erosion and release profiles was minimized. Although a number of natural and derived cyclodextrins are available, 2-hydroxypropyl-β-cyclodextrin was chosen for this project because of its low toxicity, high solubility, and wide industrial use (68, 70). The formation of imiquimod-HPβCD complexes was confirmed by DSC. Broad endothermic peak ranging from 30-

130°C observed in thermograms of pure cyclodextrin, complex and physical mixture was observed in previous studies and attributed to dehydration[18,19]. The endothermic peak of imiquimod was observed to be at little higher temperature than its theoretical melting point 292-296°C as provided by manufacturer. The absence of this endothermic peak in imiquimod-HPβCD complex plot implies changes in properties caused by the formation of complexes. The change in peak of imiquimod in the physical mixture may be explained by several reasons, such as melting point depression due to presence of cyclodextrin and its behavior as heat sink(56), amorphization of drug during the DSC run in the presence of amorphous carrier(57), and decreased intensity due to low imiquimod:cyclodextrin content (16% of total weight). The shape of the phase solubility plots again confirmed the formation of imiquimod-HPβCD complexes and suggested that imiquimod and HPβCD were more likely to form 1:1 complexes (52). Although the association constant was 23.3±1.7 M^{-1}, the final solubility of imiquimod (100 µg/mL) may not be sufficient to achieve clinically relevant dosages (i.e., 0.625 mg/cm^2, as recommended for Aldara) in the mucoadhesive film. This problem in drug-loading capacity was solved by the use of a mixture of acetate buffer (pH 4.0, 100 mM) and methanol (3:7); this mixture results in imiquimod solubility of 2.3 mg/mL. The concern that a low pH may irritate the mucosa was eliminated by the results of pH studies of the surface and the release supernatant, as previously discussed.

The high solubility of imiquimod in acetate buffer facilitated greater release (68%) of drug from polymer matrices into simulated saliva than that achieved with the other formulations. This finding was supported by a small experiment demonstrating that the addition of acetate buffer to release supernatants from other formulations (1:1 ratio) increased the measured drug concentrations (data not shown). This finding may be attributed to the hydrophobic nature of the drug, which reduces release from polymer matrices into an aqueous environment (simulated saliva). The increase accounts for the difference between acetate buffer formulations and other formulations. Regardless of the loading method, the general kinetics of imiquimod release remained similar, e.g., a small initial burst at 40 minutes followed by sustained release for 2 hours and then a small late burst at 140 to 160 minutes. The late burst may be attributed to disintegration of the swollen film into fragments, following which an increased surface area exposed to SS promoted faster release of drug. This observation suggests that the release of drug was governed by the properties of the film polymer rather than by the loading methods. In accordance with the Korsmeyer-Peppas model(45), Super-Case II relaxation was seen, and this relaxation reflects chain disentanglement and swelling of the hydrophilic films, leading to accelerated release. Although the uniformity of drug dispersion was higher with both cyclodextrin and acetate buffer formulations than with sonication formulations, films formulated with acetate buffer permitted the highest drug loading and the release of more drug because of their increased solubility; therefore, these films had an advantage over the other films.

The main purpose of solvent casting is to evaporate the solvent (water, in this project) during the drying step. The content of water remaining in a film depends on the drying time and the temperature used. Mucoadhesive film polymers hydrate on contact with water, and this hydration results in swelling followed by bioerosion. The presence of more water in the film accelerates the hydration of polymer chains deep in the film and leads to faster dissolution. The drying experiments showed that removing more water may have decreased the rate of hydration, thereby leading to slower erosion and more sustained release. The slower hydration of films may be attributed to shrinkage of gaps between polymer chains and tighter arrangement of macromolecules during the drying process. Even though films dried for more than the normal time may exhibit extended release profiles, they can be difficult to handle because of brittleness and excessive shrinkage, which frequently cause the films to curl.

The delivery system described here is intended to deliver drug locally to dysplastic mucosal lesions. The presence of a backing layer can limit the transport of drug in one direction, toward the mucosa, and can avoid direct absorption in the gastrointestinal tract. The backing layer can also improve the handling properties of the film, avoid shear forces caused by other moving parts of the oral cavity, and avoid adhesion to those other parts, such as the tongue and teeth. Treating lesions with an occlusive barrier can significantly reduce treatment periods (71, 72). In this study, parameters such as temperature, volume of toluene, and casting containers used in the making of PEVA films were optimized empirically. Any change in these parameters resulted in cracking and shrinkage of the films. Tightly sealed containers decreased the rate of evaporation of toluene, thereby allowing more time for polymers to reach equilibrium and to be properly distributed so as to form uniform films. Although films using 2.5 mL of toluene and 10% PEVA (w/v) were difficult to handle, the use of more toluene (such as 5.0 mL of toluene and 5% PEVA[w/v]) resulted in cracks. The presence of more toluene led to more evaporation, resulting in stress concentrators, shrinking, and cracking of films.

The properties of PEVA film did not change during the process of drying at 60°C. The amount of drug (2%) found in the receptor compartment showed the impermeability of PEVA. The small amount of drug released might be due to leakage of drug from the film edges that were compressed between the donor and receptor compartments under heavy force while the Franz cells were set up.

The *ex vivo* mucoadhesion time results confirmed the films' adhesive properties and their potential and advantages than Aldara cream to be retained on the buccal surface while in the dynamic oral cavity for long-term release of drug. Mucoadhesive polymers swelled substantially on contact with water and gradually began eroding with time. As the mucoadhesive polymers completely eroded, the nonadherent PEVA backing layer detached from the tissue. In contrast to expectations, decreased mucoadhesion time with increasing thickness may be attributed to excessive swelling of films. Swelling of films on contact with water increases the thickness of films as much as five fold (data not shown). Hence, the thicker films are more prone to shear and gravitational forces and thus may erode more quickly.

The current developed mucoadhesive system also involved use of ICH class 3 solvents, such as methanol and toluene. Although the amounts of these solvents were less than guidelines prescribed by FDA (38), their use may be decreased or replaced by some class 2 solvents. Imiquimod is poorly soluble in other class 2 solvents such as ethanol, but its solubility in only acetate buffer and 1:1 (methanol: acetate buffer) is 1.3 and 1.9 mg/ml, respectively. Thus, use of methanol may be decreased or avoided depending on the required drug loading. As a class 2 solvent, residual toluene in PEVA films should be minimized, such as by vacuum drying. The polymer used in the present studies had 18 wt% of vinyl acetate. Toluene may be replaced by the class 3 solvent methylethylketone using PEVA with an increased the percentage of vinyl acetate (from 18% to 40%).

3.5 Conclusion

The mucoadhesive drug delivery system described here offers a noninvasive and local approach to the delivery of an immune response modifier that may be effective in treating precancerous lesions. This drug delivery system was loaded with imiquimod, which is approved for the clinical treatment of external genital warts, basal cell carcinoma (skin cancer), and actinic keratosis and has been used off-label for mucosal disorders. Problems associated with the hydrophobic nature of imiquimod and its presence within hydrophilic polymers and aqueous environments were solved by the use of films formulated with acetate buffer. The bioavailability of imiquimod was not affected by its entrapment in a mucoadhesive polymer matrix or by all of the necessary manufacturing steps. Sustained release of the drug was achieved for three hours *in vitro*; thus, this delivery system can increase the availability of drug throughout the treatment process. Finally, a complete bilayered film, consisting of a drug-loaded mucoadhesive with a backing layer, can avoid loss of drug through the oral route and can protect the film from saliva and from the shear forces produced by the tongue, and teeth throughout the bioerosion process.

Chapter 4 Competing Properties of Mucoadhesive Films Designed for Localized Delivery of Imiquimod

4.1 Introduction

Mucosal surfaces composed of epithelial cells and connective tissues are found in many regions, such as the oral cavity, nose, eyes, and gastrointestinal, respiratory, and reproductive tracts. In addition to their natural role in protecting underlying tissues, the potential for absorption of molecules via the rich vasculature of the oral cavity makes this an attractive route for drug delivery. Delivery through oral mucosa is rapid and avoids first pass metabolism of drugs (4). Furthermore, oral surfaces offer easy access, stable pH of 6.75 compared to the stomach and intestines whose pH ranges from 2 to 7, and rapid cell recovery (10).

Several mucoadhesive delivery systems have been developed for targeting mucous membranes encompassing buccal, gastrointestinal, vaginal, ocular, nasal and sublingual surfaces (4). Although some mucoadhesive formulations were developed in 1947 (3), this field grew significantly starting in the 1980s (3, 10). The ability of these systems to adhere to mucosal surfaces increases residence time, bioavailability, provides high flux of drug, improves permeability, and retains structure of peptides and proteins (4). Commercialized systems, such as BEMA® technology (Bio Delivery Sciences International) and trans-mucosal films (Watson Pharmaceuticals), exist for systemic drug delivery but their fast erosion times (15-60 min) are not appropriate for localized treatment of diseases.

Oral squamous cell carcinoma (OSCC) is malignant form of cancer affecting squamous epithelial cells, which are present on all mucosal surfaces of the oral cavity, pharynx, and trachea. Current treatments, such as surgical resection, results in loss of tissue, which compromise normal function of the oral cavity (73). Radiation therapy of oral cancers has 100% incidence of painful post-treatment oral mucositis (74). Chemotherapy is associated with significant side effects such as myelosupression, mucositis, and hair loss, due to delivery of drug to healthy, as well as cancerous, tissues (13). A mucoadhesive system loaded with an immune response modifier, imiquimod, for potential local treatment of precancerous oral lesions was developed previously (75). Use of this delivery system would offer advantages of a non-invasive approach and reduced systemic effects of drugs.

Release of imquimod from mucoadhesive films containing polyvinylpyrrolidone (PVP) as film forming polymer and carboxymethylcellulose (CMC) as adhesive polymer was analyzed in earlier work (75). Formulations composed of 1:2 PVP:CMC were able to achieve sustained release for 3 hrs *in vitro*. Other properties relevant for developing a mucoadhesive system, such as adhesion strength, swelling, tensile properties and transport kinetics, however, were not reported. The aim of the present studies was to characterize these properties as a function of film composition and to subsequently investigate changes in drug release profiles. A more complete understanding of PVP:CMC mucoadhesives allows tuning of the system for desired drug delivery, adhesive, and mechanical properties.

4.2 Materials and Methods

4.2.1 Chemicals

Imiquimod (CalBiochem; White House Station, NJ) was incorporated into films that consisted of two polymers, polyvinylpyrrolidone (PVP) K-90 (Spectrum Chemicals; New Brunswick, NJ) and carboxymethylcellulose (CMC; sodium salt, medium viscosity; Sigma, St. Louis, MO). Other chemicals and materials used were propyleneglycol, ethanol, acetonitrile (ACN), trifluoroacetic acid (TFA), 2-hydroxypropyl-β-cyclodextrin (cell culture tested; HPβCD), poly(ethylene-co-vinyl acetate) (18wt% vinyl acetate; EVA), mucin from bovine submaxillary glands (Sigma-Aldrich; St. Louis, MO), and 15 mm Franz diffusion cells (PermeGear, Hellertown, PA).

4.2.2 Fabrication of films

Mucoadhesive films were prepared as described previously (75). Briefly, the following three solutions were prepared concurrently, thoroughly mixed, and left overnight at 43°C to remove bubbles: 1) 40% w/v aqueous solution of PVP mixed with ethanol at 1:1 v/v and followed by addition of 50% v/v propylene glycol; 2) 2% w/v aqueous solution of CMC; and 3) imiquimod solution (18 mg) using 2-hydroxy propyl-β-cylodextrin and imiquimod complexes. The polymer solutions were cast in Teflon dishes and dried at 60ºC for a time specific to the particular type of film (further presented in section 2.3). The obtained films were peeled from the dishes and stored in a desiccator at 20% relative humidity for 24 hr before use. Film formulations with varying contents of PVP and CMC were prepared as shown in Table 4.1. Blank films were used for the mechanical, adhesion, and swelling studies, while for release and transport studies, films were loaded with imiquimod. Samples (diameter, 1 cm unless otherwise noted) were punched from random points in the cast films for the following experiments.

4.2.3 Drying time

4.2.3.1 Time to achieve negligible water content.

In a pilot study, film solutions comprising 1:2 and 2:1 PVP:CMC were cast and dried at 60°C until negligible change in weight of film samples (±3%) was observed. The time required to reach this stage was recorded as steady state time (t_{st}). Because the steady state water content can vary for different ratios of PVP:CMC, the drying time to achieve equivalent contents was next identified.

4.2.3.2 Time to achieve equivalent residual water content.

Next, polymer solutions of all types of films were cast in Teflon dishes and dried at 60°C. Samples (n=3) were collected for each type of film at three specific time points ($t_{I,n}$; n=1,2,3) during the course of drying as shown in Table 4.2. After recording the weight (W_1) of the samples, they were stored in a desiccator (20% relative humidity) for 24 hr. These samples were then returned to 60°C for the t_{st} found in the pilot study (section 4.2.3.1), and the weights (W_2) were recorded. The percentage change in water content was calculated as:

$$\%\ change\ in\ water\ content = \frac{(W_2 - W_1)}{W_1} \times 100. \qquad \text{[Eq. 1]}$$

For each film composition, one time point was chosen such that all film types had equivalent water content.

Table 4.1 Different formulations of mucoadhesive films tested.

	Ratio of PVP:CMC				
	1:2	2:3	1:1	3:2	2:1
PVP (ml)	2	2.5	3	3.5	4
CMC (ml)	12	11.25	9	7.5	6
Drying time (hr)	7	8	9	10	13.5

Table 4.2 Specific drying time check points for different mucoadhesive compositions.

Ratio of PVP to CMC	1:2	2:3	1:1	3:2	2:1
Drying times (hr)	5	6	7	9	13
	6	7	8	10	14
	7	8	9	13	15

4.2.4 Tensile properties

Following removal from Teflon dishes, dog bone shaped (gauge width = 5mm, gauge length = 10mm) samples were cut from each film and fixed between the grips of a Bose ELF 3300 mechanical testing system. After preloading to 0.1 N, test specimens were deformed at a displacement rate of 3 mm/sec (14, 76, 77). The recorded load and displacement values were used to calculate the Young's modulus, ultimate tensile strength (UTS), and percentage elongation.

4.2.5 Adhesion studies

4.2.5.1 Pull-off adhesion

Porcine buccal mucosa was excised at a local slaughterhouse and frozen until use. Mucoadhesive films were attached to the moving platen of the Bose ELF 3300 using double-sided adhesive tape. Mucosal tissue was fixed to an acrylic base platen using cyanoacrylate glue. Thawed tissue was hydrated using 100 µl of simulated saliva (78) (SS; 16 mM Na_2HPO_4, 1.3 mM KH_2PO_4, 136.9 mM NaCl, pH=6.75) just before binding to a mucoadhesive film. Films were adhered to tissue with a force of 10 N for 2 mins(79) to ensure uniform binding and to imitate *in vivo* application of film to the specimen mucosa. The film was then separated from the tissue at the rate of 0.1 mm/sec (79, 80). Care was taken to use a new tissue location or new specimen for each pull-off test. Maximum adhesive force per unit area and work of adhesion were calculated from the load and displacement data.

4.2.5.2 Shear adhesion

Polycarbonate membranes (0.22µm pore size) were coated with 4% w/v bovine mucin solution and dried at room temperature overnight (59). Mucous membranes and mucoadhesive films were attached to separate glass slides using double-sided adhesive tape. After prewetting with 20 µl of simulated saliva, the two slides were adhered such that the mucoadhesive film and mucous membrane made contact under 0.5 N load for 5 mins (53). The adhered glass slides were fixed between the tensile grips of the Bose ELF 3300, and the slide bearing the mucoadhesive film was sheared away from the mucous membrane slide at the rate of 0.1 mm/sec. As for the pull-off tests, maximum adhesive force per unit area and work of adhesion were calculated from the load and displacement data.

4.2.6 Drug release and erosion studies

Based on their tensile and adhesive properties, only the 1:2 and 2:1 PVP:CMC films were further investigated. Samples were attached to the wall of 6 ml polyethylene vials to limit release of drug to only one side. These samples were immersed in SS and incubated at 37ºC with shaking at 150 rpm. Supernatants were collected and stored at predetermined intervals followed by replacement with fresh SS. Concentrations of imiquimod released into the supernatants were measured using fluorescence spectroscopy at excitation and emission wavelengths of 250 and 340 nm, respectively.

Cumulative release profiles of films were analyzed using the Korsmeyer-Peppas mathematical model (45):

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

where M_t / M_∞ is the drug fraction released at time t, k is a constant depending on structural and geometric characteristics of the system, and n is the diffusional coefficient related to release mechanism. While $n=0.45$ indicates Fickian diffusion, $0.45<n<0.89$ indicates non-Fickian diffusion, and $n>0.89$ indicates case-2 relaxation.

Erosion (mass loss) studies were performed in a similar way. The initial sample weight (W_1) was recorded before the study, and final weight (W_2) was measured after drying the degraded samples at 43ºC overnight. Mass loss was calculated and plotted against degradation time.

4.2.7 Swelling studies

Two types of swelling studies were performed on 1:2 and 2:1 PVP:CMC films and pure PVP and CMC films to further understand properties of the films.

4.2.7.1 Mass gain (conventional)

Films were attached to adhesive tape, which acted as backing layer, and incubated in SS at 37ºC. The dry weight (W_1) was recorded before immersion. Samples were then removed at predetermined intervals, blotted dry from the backing layer side, and the weights were recorded as W_2. Films were compared based on the swelling index, which was calculated as $[(W_2-W_1)/W_1]*100$.

4.2.7.2 Radial swelling

Agar plates were prepared by autoclaving 2% (w/v) LB agar in SS and then poured into polystyrene dishes. The prepared agar plates were sealed and stored upside down in the refrigerator for 2 days. After recording the initial diameter (D_1) of mucoadhesive samples, they were placed on agar plates. Diameters of samples were recorded as D_2 following predetermined intervals of incubation at 37ºC. The radial swelling index was calculated as $[(D_2-D_1)/D_1]*100$.

4.2.8 Transport kinetics and permeability characteristics

Transport kinetics of imiquimod released from films were analyzed on porcine buccal tissues that were frozen until use. Upon thawing, thin sections of 500µm thick were prepared using a sledge microtome to separate the underlying connective tissue from epithelium and used immediately. The tissue sections were mounted in a Franz cell such that epithelial side faced the donor compartment. Mucoadhesive samples were applied to the mucosal surface of tissue contained in the Franz cell. After filling the receptor compartment with 12 ml of SS, care was taken to ensure the tissue surface was always in contact with the solution.

Supernatant was collected from the receptor compartment at predetermined intervals and replaced with fresh SS. Acetate buffer (100 mM, pH 4.0) was freshly prepared and added at a ratio of 50:50 (v/v) to all collected samples to solubilize drug before measurement. Experiments were run for 24 hr, after which residual film was solubilized completely in 50:50 acetate buffer:SS to quantify the remaining drug. Tissue sections were also immersed overnight in 50:50 acetate buffer:SS to extract retained imiquimod. The amount of drug in all samples was determined by reverse phase high performance liquid chromatography (HPLC) using a Shimadzu Prominence system equipped with a Phenomenex C_{18} column. The mobile phase used was 40:60 ACN to water containing 1%TFA at a flow rate of 1 ml/min. Imiquimod was measured using a UV detector at a wavelength of 242 nm.

Permeability and transport kinetics of control solutions (imiquimod solubilized in acetate buffer) and imiquimod-loaded 1:2 and 2:1 films were compared. Care was taken to ensure that mucoadhesive films and control solutions had equal amounts of drug (0.26 mg). The cumulative amount of drug permeated through tissue per unit area was calculated and plotted as a function of time. Flux (Q) of drug was then calculated from the slope of the linear portion of the curve.

4.2.9 Statistical analysis

All experiments were conducted in triplicate and repeated at least once to demonstrate reproducibility of results. The results are expressed as mean ± standard deviation. While unpaired two-tail student t-tests were used to compare instantaneous release of drug, degradation and swelling, ANOVA with the Tukey post-hoc test was used for tensile, adhesive studies, and transport kinetics and permeability studies. Results were considered statistically significant if $p<0.05$.

4.3 Results

4.3.1 Drying time

Film samples (1:2 and 2:1 PVP:CMC) were observed to lose water when dried for extended periods of time. Although significant changes were observed through the first 5 hr, mass decreases slowed thereafter; negligible change (±3%) was observed between 11 and 24 hr for all types of films. Hence, 24 hr was chosen as the steady state drying time point (T_{st}) at which minimal water content of films was achieved.

After determining that maximal water loss occurred by 24 hr, fresh mucoadhesive films were prepared by drying for different times ($t_{I,n}$). Samples were then punched and dried again for 24 hr (t_{st}) to find the water content. Irrespective of film type, all samples lost water, and as expected, the percentage change in water content decreased with increased initial drying time ($t_{I,n}$) (Figure 4.1). Based on these observations, drying times for 1:2, 2:3, 1:1, 3:2, and 2:1 films were chosen to be 7, 8, 9, 10, and 13.5 hr, respectively, to achieve a uniform water content of 39±2.5%. Smooth, bubble free, and flexible films of each type were obtained after drying for their respective times. Tackiness of films increased with increasing PVP content of films, which made handling slightly difficult.

4.3.2 Tensile properties

The stress–strain curves of all film types, except those with 2:1 PVP:CMC, showed two slopes before failure (Figure 4.2a); the initial slope was used to calculate elastic modulus. As shown in Figure 4.2b, both elastic modulus and UTS decreased significantly ($p<0.0001$) with increasing PVP content. Elastic modulus of films ranged from 6.9±1.5 to 1.8±0.2 MPa and UTS ranged from 4.2±0.7 to 2.1±0.02 MPa for 1:2 and 2:1 films, respectively. Percentage elongation, however, significantly ($p<0.0001$) increased with PVP content, ranging from 129.2±13.5 to 394±47.3 % for 1:2 and 2:1 films, respectively (Figure 4.2b). A detailed presentation of significant differences between each type of film is shown in Table 4.3.

Films became soft, tacky and viscoelastic as PVP content increased. This viscoelastic behavior was more evident in 3:2 and 2:1 film types in the form of strain recovery. When films were elongated until just before breakage (x) and returned to half of that elongated length (x/2), the films were observed to recover from this strain in less than 30 seconds (final length of films = initial length + x/2) as shown in Figure 4.3.

4.3.3 Adhesive properties

Detachment of samples in both pull-off and shear adhesion studies occurred only at the interface between polymer and tissue/mucin. The average maximum adhesive strength (force per unit area) required to detach mucoadhesive films from porcine buccal tissue increased significantly with PVP content ($p<0.0003$) from 0.42±0.03 to 1.1±0.1 N/cm^2 for 1:2 and 2:1 mucoadhesive films, respectively (Figure 4.4a). Increasing PVP content, however, did not significantly affect work of adhesion (Figure 4.4a). For shear adhesion, both the maximum shear force and work of adhesion required to peel mucoadhesive films from mucin-coated membranes significantly increased with PVP content ($p<0.0001$) (Figure 4.4b). The maximum shear adhesive strength increased ($p<0.0001$) from 1.7±0.25 to 5.6±1.4 N/cm^2 and work of adhesion increased ($p<0.0001$) from 4.3±1.1 to 12.9±0.84 N/cm^2 for 1:2 and 2:1 mucoadhesive films, respectively. A detailed presentation of statistically significant differences between each type of film is shown in Table 4.3.

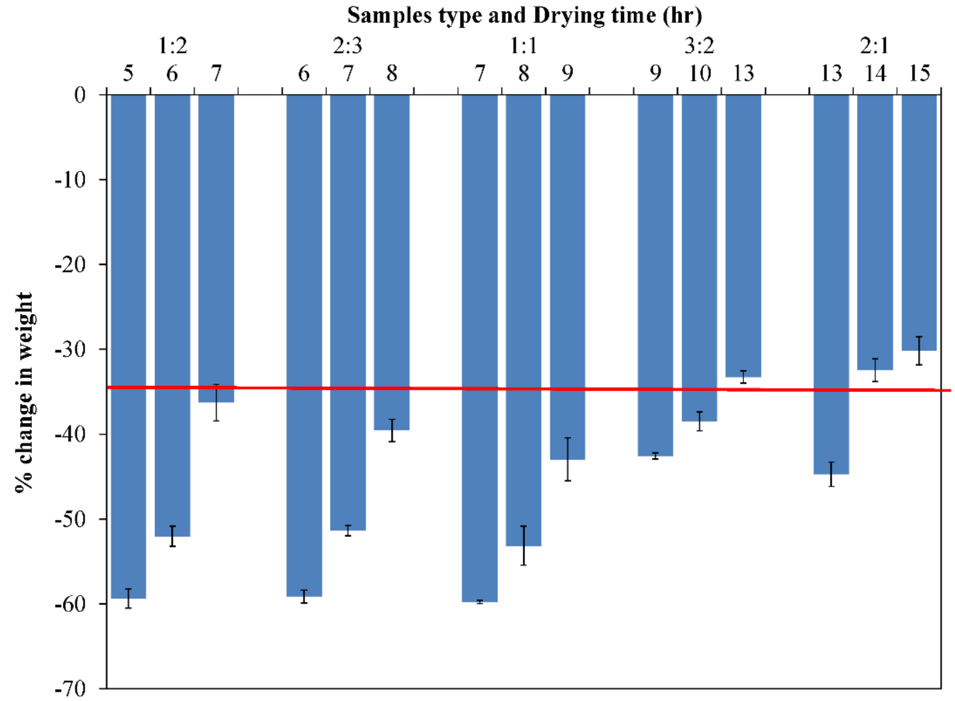
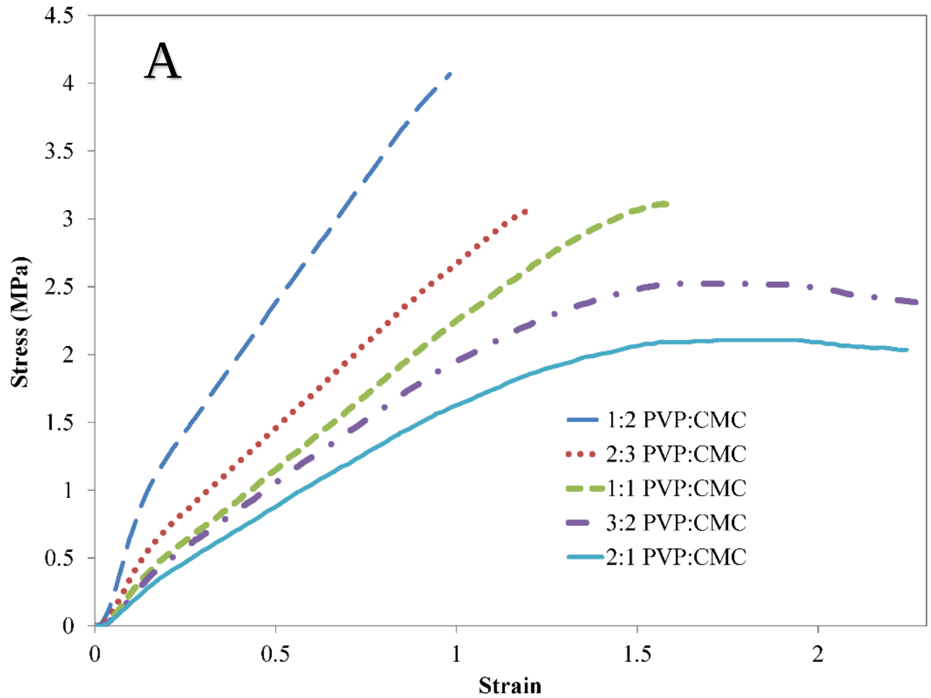


Figure 4.1. Percentage change in weight of mucoadhesive films at different initial drying times. Data are mean ± standard deviation (n≥3). Initial drying times were chosen such that all films had equal water content of 39% (red line).

Table 4.3. *Post hoc* statistical results from the tensile and adhesion studies (Figures 2B and 4).

Sample	Modulus	UTS	% elongation	Max pull-off adhesive strength	Max shear adhesive strength	Shear work of adhesion
1:2 vs 2:3	ns	ns	ns	*	ns	ns
1:2 vs 1:1	***	*	*	*	ns	ns
1:2 vs 3:2	***	***	**	**	ns	*
1:2 vs 2:1	***	***	***	***	***	***
2:3 vs 1:1	**	ns	ns	ns	ns	ns
2:3 vs 3:2	**	**	*	ns	ns	ns
2:3 vs 2:1	***	***	***	*	***	**
1:1 vs 3:2	ns	ns	ns	ns	ns	**
1:1 vs 2:1	ns	*	***	*	***	***
3:2 vs 2:1	ns	ns	***	ns	**	ns

*ns = not significant ($p<0.05$); * = ($p<0.05$); ** = ($p<0.01$); *** = ($p<0.001$);



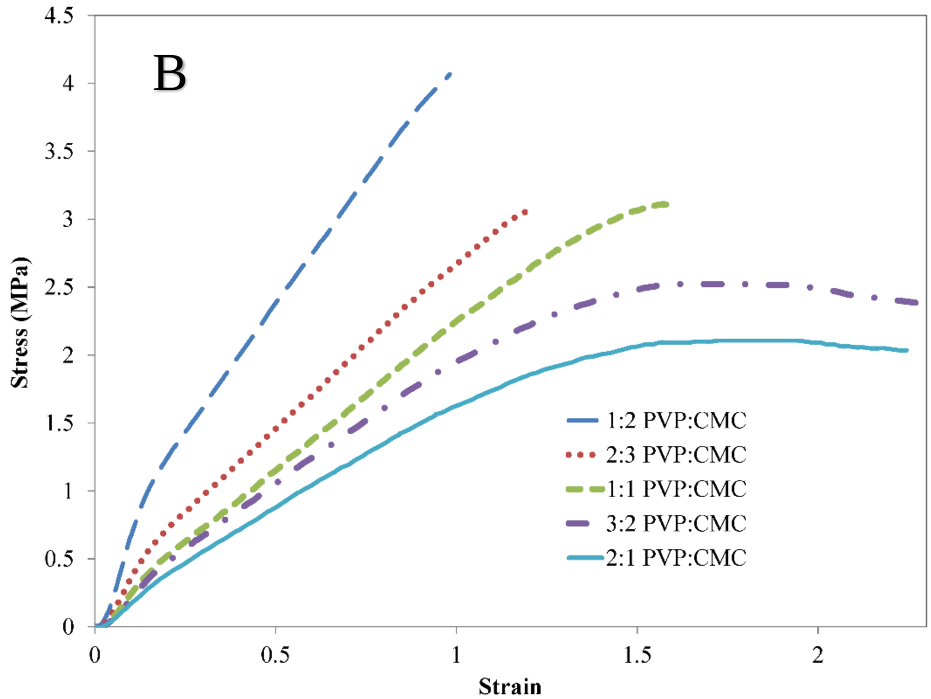


Figure 4.2. A) Typical stress-strain curve of films with two different slopes. A representative plot for a 1:2 PVP:CMC film is shown. B) Modulus, UTS, and percentage of elongation of all mucoadhesive film compositions. Results of statistical analysis are shown in Table 4.3. Data are mean ± standard deviation (n≥3).

4.3.4 Drug release, erosion, and mathematical modeling

The 1:2 PVP:CMC films were able to achieve sustained release of imiquimod for up to 3 hr with a burst at 160 min and continuously decreasing release thereafter (Figure 4.5). For 2:1 films, however, most of the imiquimod was released over the first hour, after which release continually decreased over time (Figure 4.5). Cumulative release profiles for both types of films coupled with their respective mass loss profiles are shown in Figure 4.6. For 1:2 PVP:CMC, the profile of drug release closely followed erosion of the films (Figure 4.6a). The 2:1 films, however, began eroding early and eroded faster (Figure 4.6b); their initial mass loss was greater than the percentage of drug released up to 40 min. In addition, imiquimod release from 2:1 PVP:CMC films occurred faster than from 1:2 films. Inconsistencies in the last few time points of the erosion experiment measurements were attributed to difficulties in handling of the viscous and mostly eroded films. Mathematical modeling of release profiles based on the Korsmeyer-Peppas equation showed 'n' values of 1.03 for 1:2 PVP:CMC films and 0.89 for 2:1 PVP:CMC films.

4.3.5 Swelling

Conventional swelling studies indicated rapid mass changes, which resulted in indices reaching up to 500 and 200 for 1:2 and 2:1 PVP:CMC films, respectively (Figure 4.7A). The 1:2 PVP:CMC films swelling indices reached 200 in the first five min and 400 at 60 min. The rate of swelling subsequently decreased, and a further increase of only 100 was observed over the next 90 min. In contrast, 2:1 PVP:CMC films reached swelling index of 200 by 40 min and started decreasing from 60 min by losing mass. Although both types of films became extremely viscous and difficult to handle after 130 min, 1:2 films maintained their integrity, unlike 2:1 films, which started eroding as observed visually. Swelling indices of the films were significantly different ($p<0.05$ to $p<0.001$), except at the first time point of 10 seconds.

In contrast to the conventional swelling studies, measurement of radial swelling on agar showed that 2:1 PVP:CMC films swelled more than did the 1:2 PVP:CMC films (Figure 4.7A).The swelling indices of both films were less than 100, but both films continued to swell radially after 180 min, unlike the conventional mass gain studies in which swelling plateaued and the samples began losing mass. At longer times, loss of the samples circular shape made further measurements difficult. Swelling indices of both films were significantly different ($p<0.05$ to <0.0025), except at the first time point of 15 min.

Conventional swelling profiles for films containing only CMC showed a high index of 3000 in 150 min with monotonically increasing swelling. Unlike CMC films, PVP only films reached their maximum swelling index of 264 in 45 min and then quickly started losing mass, being completely eroded by the end of 150 min (Figure 4.7B).

4.3.6 Transport kinetics and permeability

Imiquimod in all samples was successfully separated from tissue particles and polymer components using HPLC. While imiquimod had a sharp peak at the retention time of 3.3 min, solubilized molecules of tissue had a broad peak 3.8 min; PVP and CMC were found with the injection peak. Imiquimod was detectable within a linear concentration range from 60 ng/ml to 7.8 µg/ml.

Transport of imiquimod through porcine buccal tissue into simulated saliva was controlled by 1:2 and 2:1 mucoadhesive films. The average flux rates of imiquimod through buccal mucosal tissue were 1.25±0.39, 1.11±0.12, and 4.98±0.91 $\mu g/cm^2/hr$ for 1:2 and 2:1 mucoadhesive films and the control solution, respectively (Figure 4.8A). The mucoadhesive films significantly ($p<0.01$) decreased flux of imiquimod through tissue compared to control solutions, while no significant difference in flux was observed between 1:2 and 2:1 film types. Imiquimod retained in tissue after 24 hr increased with the use of mucoadhesive films (30%) and was observed to be double the amount of imiquimod retained from control solutions (15%) (Figure 4.8B). The amount of imiquimod transported through tissue into simulated saliva was 3.5 fold higher for control solutions compared to when films were used ($p<0.001$).

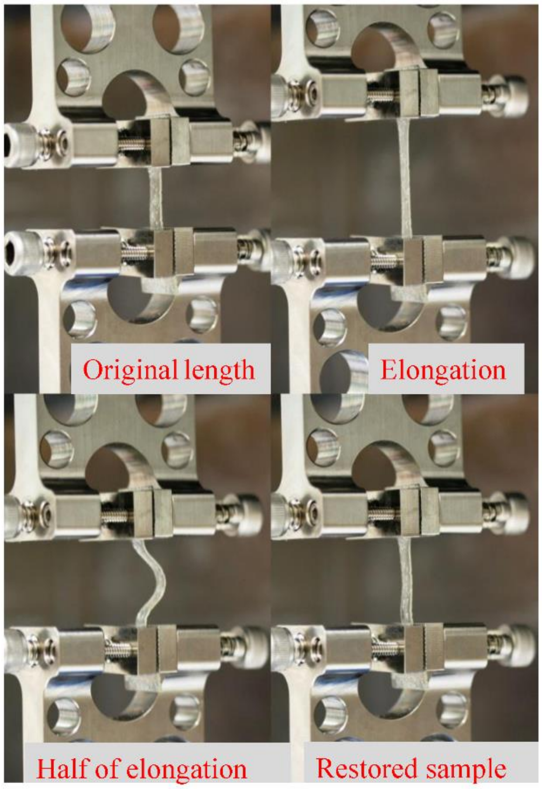


Figure 4.3. Strain recovery behavior of 2:1 PVP:CMC film. When film was deformed and then returned to its original length, the polymer chains rearranged to recover the initial deformation.

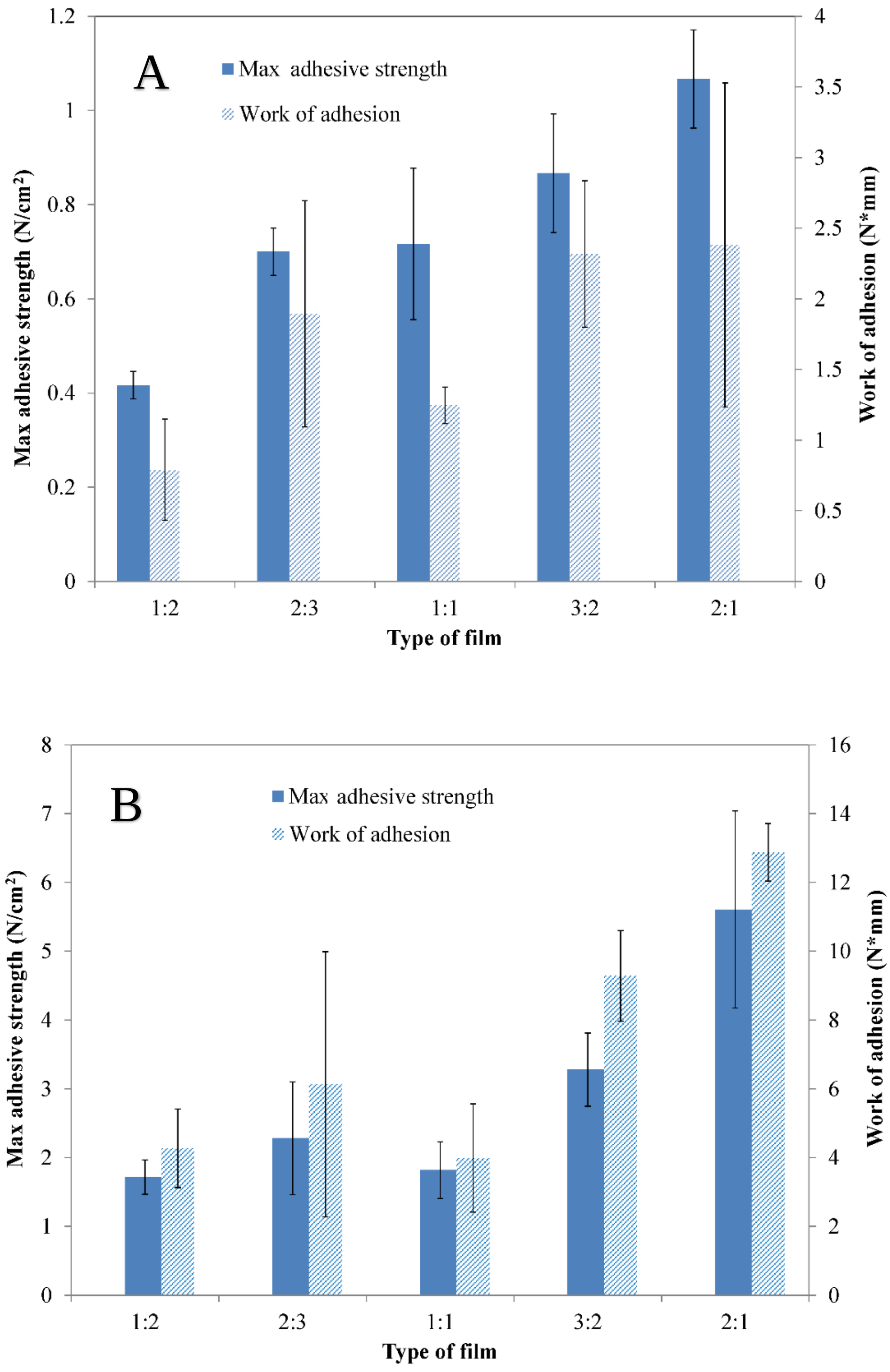


Figure 4.4. A) Maximum pull-off adhesive strength (force/unit area) and work of adhesion for films on porcine buccal tissue. B) Maximum shear adhesive strength (force/unit area) and shear work of adhesion for films on mucin-coated membranes. Results of statistical analysis are shown in Table 4.3. Data are mean ± standard deviation (n≥3).

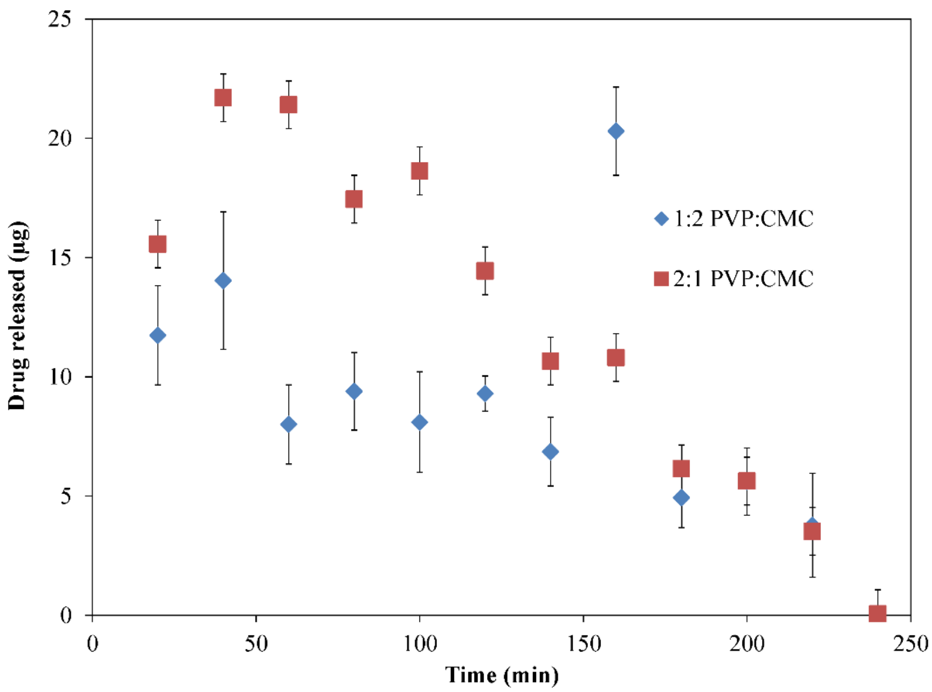
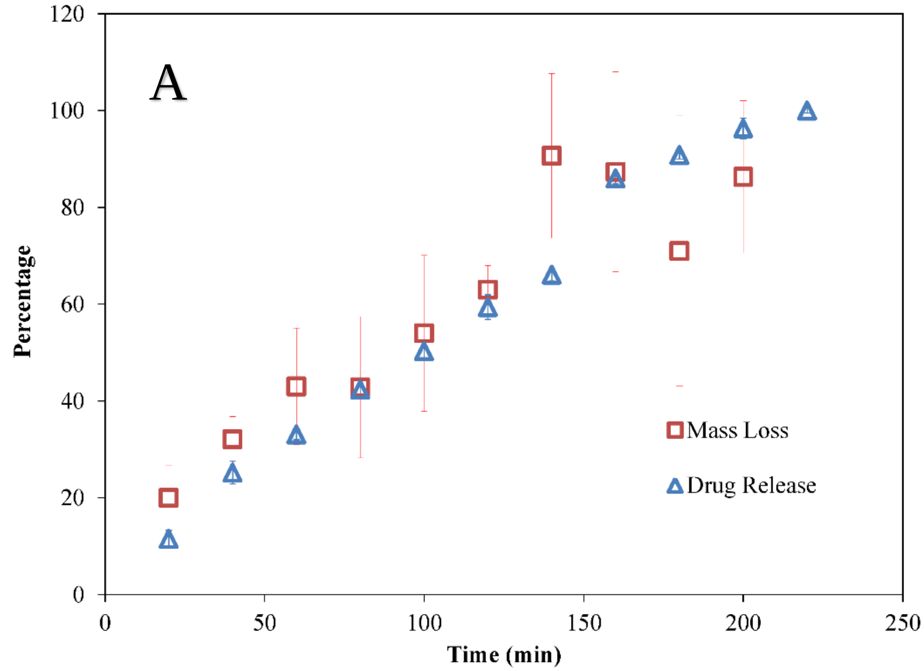


Figure 4.5. Instantaneous release of imiquimod from 1:2 and 2:1 PVP:CMC films. Data are mean ± standard deviation (n≥3).



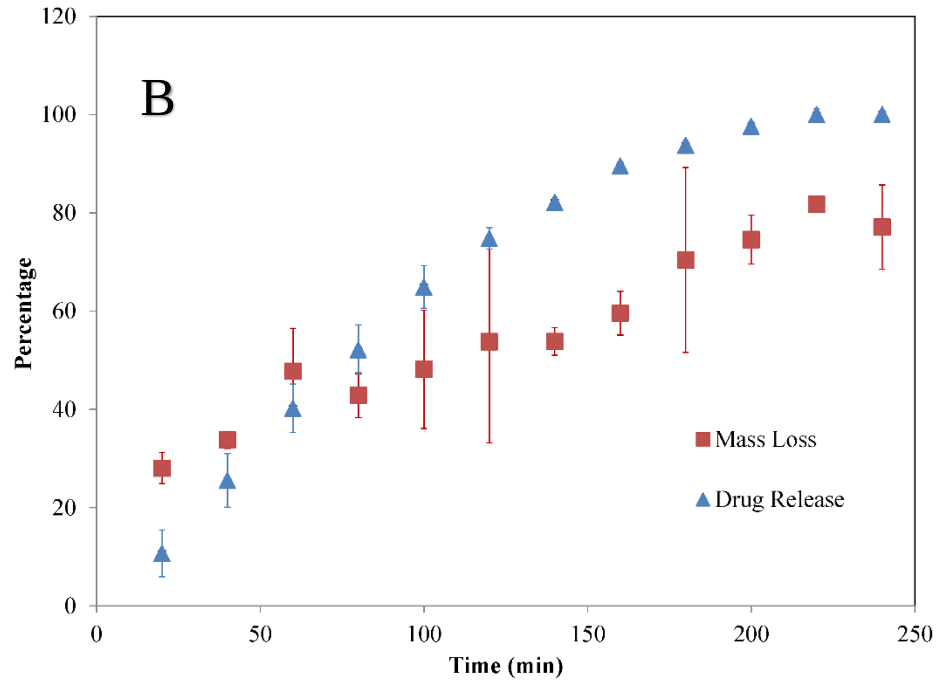


Figure 4.6. Cumulative imiquimod release profile coupled with erosion profile for A) 1:2 and B) 2:1 PVP:CMC films. Data are mean ± standard deviation (n≥3).

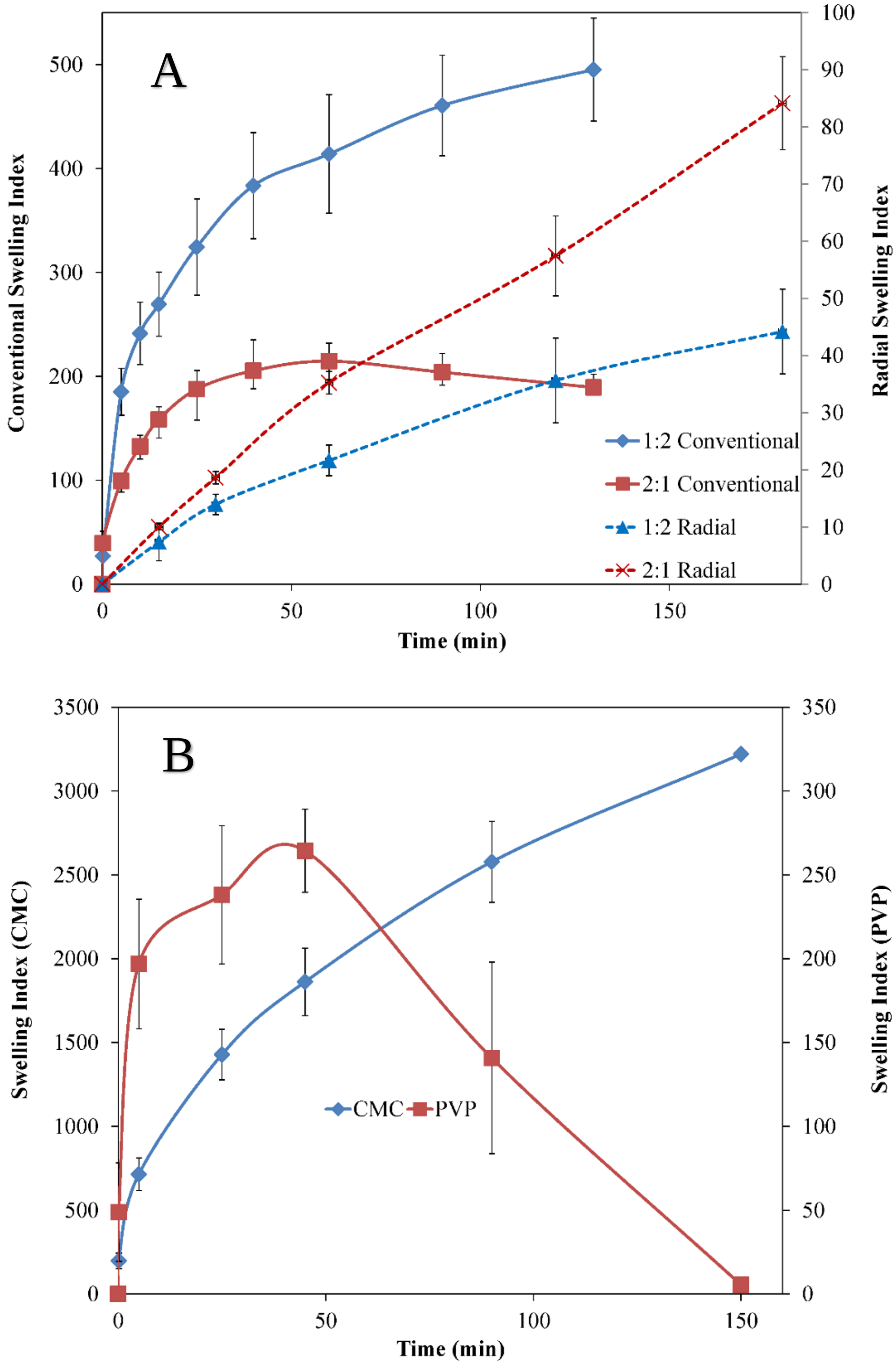


Figure 4.7. A) Conventional and radial swelling profiles for 1:2 and 2:1 PVP:CMC films and B) conventional swelling profiles for pure PVP and CMC films. Data are mean ± standard deviation (n≥3).

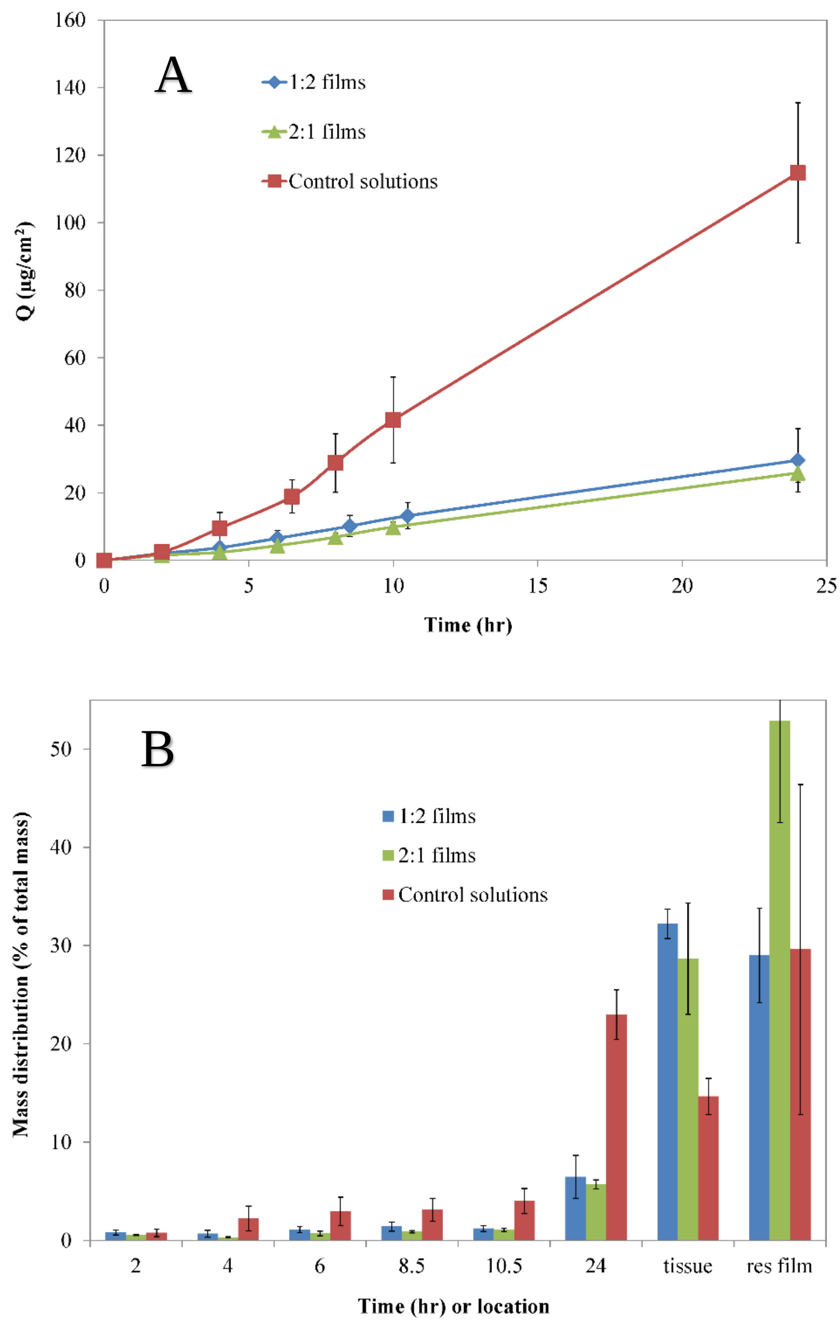


Figure 4.8. A) Cumulative drug permeation per unit area vs. time for control solution (imiquimod solubilized in acetate buffer) and PVP:CMC mucoadhesive films. B) Percentage of total amount of imiquimod found in receptor compartment at increasing times, retained in tissue, and residual (res) film after 24 hr. Data are mean ± standard deviation (n≥3).

4.4 Discussion

Mucoadhesive films loaded with immune response modulators can provide a local and non-invasive approach to treatment of oral precancerous lesions. Although previous work (75) showed that 1:2 PVP:CMC mucoadhesive films lasted 4 hr submerged in sink conditions *in vitro* and achieved sustained release for up to 3 hr, several other film properties were not reported, such as adhesiveness of film to mucous surface, mechanical properties for better handling and swelling, which determines the release mechanism. All of these characteristics will play key roles in the design of a successful bioerodible, mucoadhesive drug delivery system. The versatility of the present delivery system allows modification and tailoring of these properties by simply changing composition of the films.

The film fabrication process involves drying polymer-drug solutions at 60°C to evaporate the remaining solvent, mainly water. The amount of solvent retained in the films depends on the drying time and film composition. Previous work (75) with this delivery system showed that residual water content significantly affected the drug release profile. Qualitative observation of films also showed differences in tackiness, strength, and flexibility with varying water content. Hence, it was important to control this variable for an accurate comparison of other film properties. Based on an analysis of mass changes as a function of time, the drying time for all film compositions was selected to maintain around 39% of residual water content in films. Further drying of films resulted in loss of flexibility and brittleness, and under-drying of films results in a soft gel that was difficult to handle.

Even though mucoadhesive films do not have load-bearing responsibility, understanding their tensile properties may be useful for better handling of films during the manufacturing process, while being applied to a mucosal surface, and when exposed to potentially demanding *in vivo* conditions. Selection of 3 mm/sec deformation rates was based on previous work with mucoadhesive films in which deformation rates ranged from 1 to 5 mm/sec (14, 76, 77). Modulus (1.8 to 6.8 MPa) and tensile strength (2.1 to 4.2 MPa) of the PVP:CMC films decreased with increasing PVP content, which was contrary to results expected from PVP being considered the film forming polymer (15). While the observed tensile strength and modulus were comparable to mucoadhesive films prepared from chitosan/copolymer of polyvinyl alcohol (PVA)/polyethylene glycol (PEG) (UTS =

3.5 to 5.4 MPa and modulus = 2.7 to 7.5 MPa) (16) and copolymer of methylvinylether and maleic anhydride (UTS = 2.77 MPa) (14), the UTS of PVP:CMC was 10 times lower than that for films made of hydroxypropylcellulose (20 to 110 MPa) and hydroxypropylmethylcellulose (40 to 150 MPa). The present mucoadhesive films exhibited substantial elongation before failure (150-300%) compared to 12-35%, 55-125%, and 66-130% reported for hydroxypropylcellulose, hydroxypropylmethylcellulose, and chitosan/PVA/PEG films, respectively (16, 81).

Pull-off adhesion studies were performed on porcine buccal mucosa because of its resemblance to human buccal mucosa in terms of ultrastructure and composition (12, 82). An initial force of 10 N was applied on films to imitate pressing a film onto a patient's cheek by a finger, as well as being based on similar work (79). Substantial variability was observed during pilot testing of multiple samples on a single tissue specimen. This was likely due to microscale adhesion of polymers on tissue and/or components of the tissue surface being modified with each test. Hence, care was taken to use fresh location on same tissue or a fresh tissue for each sample to reduce variability. Comparison of the present results with existing literature was difficult due to different experimental conditions, such as contact force, deformation rate, and contact time. The measured mucoadhesive forces, however, were comparable to several blends of polymers (15, 16, 83, 84). While the adhesion force increased from 0.41 to 1.06 N/cm^2 with increasing PVP content, several other polymer blends, including chitosan/PVA/PEG ranged from 0.33 to 0.41 N/cm^2 (16); copolymers of acrylic acid and 2-ethylhexyl acrylate ranged from 0.033 to 0.065 N/cm^2 at different contact speeds and contact times (83); and plain films of hydroxyethylcellulose, chitosan and polyvinyl alcohol recorded 0.58, 0.88 and 5.11N/cm^2, respectively (84).

Shear stresses from the tongue, gums, and saliva may be more prominent than pull-off forces under actual oral conditions. The Wilhelmy plate method is commonly used to measure shear adhesion of polymers, often with mucin solution instead of tissues (4). Buccal mucosa is covered by mucus, which contains 4% mucin (glycoproteins) (11). Several studies have proposed that the rate of diffusion of polymer chains into mucus and their interactions with mucin are the main factors responsible for mucoadhesion of polymeric films (2, 10, 11). Hence, 4% bovine mucin-coated membranes were used as replacement of porcine buccal tissues for shear adhesion studies.

Variables, such as contact force, contact time, and amount of buffer used to hydrate, play important roles in the performance of films in shear adhesion studies (53, 83). Consequently, the parameters used for the adhesion experiments were based on pilot studies (data not shown). The difference in contact forces for pull-off and shear adhesion was primarily attributed to the change of substrate. Furthermore, films were observed to tear during shear adhesion studies following application of 10 N contact force rather than desired sliding of films on mucin-coated membranes, which was why the initial contact force was reduced. However, both pull-off and shear maximum adhesive force per unit area were observed to increase with increasing PVP content, although CMC is well known as a mucoadhesive polymer (85).

Changes in both mechanical and adhesive properties showed clear trends with changing PVP content. Because measurements of mechanical properties showed that 1:2 films were tough with high modulus and UTS and that 2:1 films were more adhesive, only 1:2 and 2:1 PVP:CMC films were selected for better understanding of PVP and CMC effects on release, swelling, and erosion profiles. The close relationship between drug release and mass loss for 1:2 films suggests that release of imiquimod was controlled by erosion of the films. Comparison of mass loss and release profiles for 2:1 films, however, suggests that release of imiquimod was controlled by both diffusion and erosion. This interpretation was also supported by Korsmeyer-Peppas mathematical modeling. According to this model 'n' value of greater than 1 suggests super case-2 relaxation, which involves erosion of films and swelling-controlled polymer relaxation, and the 'n' value of 0.89 suggests anomalous, non-Fickian diffusion.

Two types of swelling studies were performed for better understanding of film behavior. While conventional swelling studies based on mass gain, the gold standard for swelling studies, showed behavior of films in bulk solutions, the agar-based radial swelling studies can better mimic the conditions of a film applied to the mucosal surface, which is the intended application of this delivery system. CMC, which is known for its water-retaining properties, was observed to reach swelling index of 3000 and still retain its integrity, unlike PVP films, which had a lower swelling index and eroded faster. Hence, the presence of more CMC in 1:2 PVP:CMC films caused more swelling compared to 2:1 PVP:CMC films. Early erosion of 2:1 PVP:CMC films at 60 min can also be attributed to the presence of more PVP. This early erosion of polymer chains from 2:1 PVP:CMC films may have enhanced diffusion of eroded chains through agar, resulting in more radial swelling than was observed for 1:2 PVP:CMC films.

Combining both mathematical modeling, release, erosion and swelling profiles, it can be understood that drug release from 1:2 PVP:CMC films was controlled by swelling and slow erosion of films that resulted in sustained release. In contrast, earlier and faster erosion of chains and less swelling opened up the bulk of 2:1 PVP:CMC film and resulted in burst release. This was then followed by continually decreasing concentrations of drug, which were governed by diffusion of drug from the residual polymeric matrix into buffer.

Because a potential application of the mucoadhesive films is for local treatment of oral dysplasia, studying the transport characteristics and permeability of imiquimod *in vitro* can give preliminary knowledge about feasibility of this approach before initiating *in vivo* studies. The goal is to deliver and retain drug in the epithelium rather than penetration into vasculature for systemic distribution. Porcine tissue was chosen because of its close resemblance to human buccal mucosa and its extensive use in other permeability studies (12, 82). The thickness of epithelium in human mucosa ranges from 250 µm to 400 µm (12) . Prior studies showed that use of tissue sections ≤500 µm represents transport kinetics of a compound through epithelium. Transport kinetics through mucosa were dominated by connective tissue when tissue sections were >500 µm (12).

Permeability studies of four different compounds encompassing hydrophilic and hydrophobic compounds on only the epithelial layer showed that permeation increased with lipophilicity (12). Other permeability studies on hydrophilic substances, such as mannitol and lidocaine hydrochloride, showed low permeability and used fatty acids, such as oleic acid, to increase the permeation through the epithelium (86, 87). Hydrophobic susbtances, such as carvedilol, had rapid permeation in the first few hrs (81). Because imiquimod is also hydrophobic, it showed good permeability when used alone in a solution. The current PVP:CMC mucoadhesive films significantly decreased the flux of imiquimod and helped localize imiquimod in the epithelium. Interactions of mucoadhesive polymers with the epithelial tissue as well as the hydrophilicity and large size of the polymers may have resulted in their being trapped in the tissue, which created a transport barrier and reduced permeation. Brief literature review of several other mucoadhesive systems for the above localization effect of drug revealed absence of the above type of data. Majority of articles didn't compare/ report/analyze the amount of drug being retained in tissue when films and control solutions were used (45, 61, 81, 86, 88, 89). Parameters such as flux and permeability coefficient were only reported. Although 1:2 PVP:CMC films exhibited sustained release of imiquimod for up to 3 hrs in contrast to 2:1 films, for which burst release was observed in first 40 mins and continously decreased release, no significant difference was evident between both films in transport kinetics or absorption within epithelium. This may be attributed to inability of the polymers to permeate the tissue, which thereby acted as the rate limiting step, rather than erosion of polymers, which control the release of drug.

A variety of polymers are being used to develop mucoadhesive films for delivery of different drugs. Some of the more extensively used polymers include hydroxypropylmethylcellulose (HPMC), chitosan, hydroxylethylcellulose (HEC), carbopol, Eudragit RL PO, gelatin, CMC, PVA, polyethylene (PE) and PVP (K30 and K90 variations) (13-16, 45, 60, 61, 81, 83, 84, 86, 90). In addition, new copolymers are being developed, such as copolymers of methylvinylether and maleic anhydride (PMVE/MA) (14) and acrylic acid and 2-ethylhexyl acrylate (83). All these polymers and blends of different compositions have advantages and disadvantages. For example, while chitosan is a natural, adhesive polymer, the resulting films can be brittle (60). Addition of other polymers, such HEC (60), PVP K30 (13), copolymer of PVA and PE (16), to chitosan increased the film-forming ability, UTS, and percentage of elongation. In the present work, however, a range of properties can be achieved simply by adjusting the ratio of PVP to CMC.

The measured range of adhesion, mechanical, and drug release properties of mucoadhesive films can be attributed to the combined properties of PVP and CMC. The hygroscopic nature and tackiness of PVP increased adhesive properties, while the excessive swelling and slower erosion of CMC aided film retention during the release studies. The ability of PVP to absorb moisture (up to 40% of its weight) resulted in decreased modulus and UTS because it enabled PVP chains to move and reposition more easily under load. With increasing CMC content, however, chain entanglement and decreased mobility may have increased modulus and UTS and decreased elongation. Swelling studies showed the early erosion of pure PVP films beginning at 60 min, but the presence of CMC helped control erosion, thereby providing sustained release for 3 hr.

4.5 Conclusion

The present mucoadhesive drug delivery system based on CMC and PVP offers a wide range of tensile, adhesive, degradation, and release properties without addition of new polymers/excipients. Controlled release and increased localization of imiquimod within the epithelium provided by PVP:CMC mucoadhesive films may increase the potential of these films for local treatment of oral dysplasia. Further bioactivity studies *in vivo* will be important for determining the best combination of properties and appropriate film type for treatment of dysplastic lesions

Chapter 5 Effects of Epidermal Growth Factor-Loaded Mucoadhesive Films on Wounded Oral Tissue Rafts

5.1 Introduction

Craniomaxillofacial (CMF) injuries encompassing hard and soft tissues occur in 15-34% of general trauma patients (91), the epidemiology of which involves accidents, sports, and violence (30). Battlefield casualties also include significant CMF trauma. In Operation Iraqi Freedom, 26% of injuries involved CMF, and 58% of those had soft tissue (mucosal and connective tissue) deficits (31). Oral mucosal deficiencies are also created in several clinical conditions, such as post-neoplastic ablation, periodontal pathologies, tooth replacements, and preparation of oral mucosal grafts for urethral reconstructive surgery (32, 33).

The gold standard for treating defects in the oral mucosa is use of autologous tissue from other mucosal surfaces (91). This approach, however, has several shortcomings, including limited availability of mucosal tissue for harvest and second surgical site morbidity. Although autologous skin grafts have also been used, several complications, such as excessive keratinization, absence of a moist surface at the recipient site, and unwanted hair growth, were observed (32). Tissue engineered oral mucosal grafts grown *ex vivo* are being explored as an alternative treatment. Some of these grafts, such as those obtained by culture of epithelial cells derived from the stem cells of patients, or collagen scaffolds (obtained by decellularization of cadaveric dermis (Alloderm®)) loaded with epithelial cells, improved healing and showed short-term clinical success (34, 35). Such tissue engineered grafts, however, still lack key morphological characteristics, such as a thick epithelium, rete ridge formation, and a mature basement membrane (32). In addition, they can also be limited by unavailability as off-the-shelf products, reduced viability, difficult manipulation and handling during surgery (35), and cost of the treatment. Although tissue grafts made from a patient's own cells can be useful in preplanned surgeries, they may not be available in trauma situations for 4-6 weeks. A readily available system/biologic capable of protecting the wound site and promoting native mucosal regeneration may significantly improve the healing process.

Mucoadhesive drug delivery systems are being used to improve the efficiency of drug administration and avoid first pass metabolism (3, 4, 10). In addition to protecting a wound from the oral environment, these systems can provide controlled release of drug with high flux and increased bioavailability (4). Commercialized systems, such as BEMA® technology (Bio Delivery Sciences International) and trans-mucosal films (Watson Pharmaceuticals), are primarily used for rapid systemic drug delivery. These systems having short erosion times (15-60 min) are not appropriate for localized treatment of conditions requiring prolonged availability of drug.

Epidermal growth factor (EGF), a polypeptide of 53 amino acids, plays an essential role in epithelial wound healing by stimulating proliferation and migration of keratinocytes. It also promotes formation of granulation tissue and stimulates fibroblast motility (92). *In vitro* studies show that EGF plays a significant role in healing of mucosal tissues by promoting migration and proliferation of epithelial cells in the oral cavity and intestinal mucosa (93, 94). Exogenous EGF improved tongue wound healing in sialodenectomized mice, *i.e.*, mice from which salivary glands were removed, where the majority of EGF is produced (95). Elevated levels of EGF were found in humans for up to 48 hours after oral surgery, which promoted faster healing (96).

EpiOral (ORL 300-FT; MatTek, Ashland, MA) is a lab-grown, multilayered, epithelial tissue supported by a collagen bed containing fibroblasts, which is similar in structure to oral mucosa and submucosa. These *in vitro* buccal phenotype tissues grown from human oral keratinocytes exhibit several important features, such as lipid profile, basal cells, human beta-defensins, metabolic and mitotic activity, and several specific integrins. ORL 300-FT tissues are also being used in irritation, oral pathology (mucositis), and absorption studies as an alternative to animal testing (97). The similar structure to native tissue and presence of active cells capable of producing biomolecules and proliferating provide a unique platform for investigating mucoadhesive films in comparison to *in vitro* testing in sink conditions (simulated saliva) or with nonviable tissues *ex vivo*. Intimate contact, rate of swelling, erosion, clearance of polymers, and interactions with cells can better mimic *in vivo* conditions.

Owing to the key role of EGF in promoting mucosal wound regeneration and the advantages of mucoadhesive delivery systems, the goal of the current studies was to develop a mucoadhesive system providing sustained release of bioactive EGF. Because application of mucoadhesive films in the oral cavity of small animals poses practical difficulties, the efficacy of the materials was subsequently investigated *in vitro* using buccal tissues (ORL 300-FT) as a potential replacement for small animal studies.

5.2 Materials and Methods

5.2.1 Fabrication of films

Mucoadhesive (1:2 PVP:CMC) films were prepared from polyvinylpyrrolidone (PVP) K-90 (Spectrum Chemicals; New Brunswick, NJ) and carboxymethylcellulose (CMC; sodium salt, medium viscosity; Sigma, St. Louis, MO) (98). Briefly, solutions of PVP (40% w/v aqueous solution of PVP combined with ethanol at 1:1 v/v and followed by addition of 50% v/v propylene glycol) and CMC (2% w/v aqueous solution) were mixed. Human recombinant EGF (Shenandoah Biotechnology, Warwick, PA) or lysozyme (Sigma) solubilized in deionized water containing 0.1 % bovine serum albumin (BSA; Sigma) was then added to the polymer solution, thoroughly mixed using a heavy duty rotator at high speed (Roto Torque; Cole Parmer, Chicago, IL), and left overnight at 43°C to remove bubbles. The resulting mixture was cast in Teflon dishes and dried at 60ºC for 5.5 hours. The obtained films were stored in a desiccator at 4ºC with 20% relative humidity for 24 hours before use.

5.2.2 Release studies

The difference in release profiles between sink and non-sink conditions was investigated using mucoadhesive films loaded with lysozyme (4 µg). Profiles for EGF-loaded films were then determined in non-sink conditions. Samples contained 105 ng of EGF to achieve and maintain a concentration of 3.5 ng/mL in the release supernatants. Concentrations of lysozyme labeled with AlexaFluor 350 (Invitrogen, Carlsbad, CA) were determined by measuring fluorescence (excitation=346 nm, emission=442 nm), and EGF was quantified by ELISA (Fisher Scientific, Waltham, MA).

For sink conditions, samples of 10 mm diameter were attached to the wall of 6 mL polyethylene vials so that release of drug was limited to only one side. All polyethylene vials were precoated with 0.1% BSA to block nonspecific adsorption sites and limit the loss of protein. The samples were immersed in 6 mL of simulated saliva (SS; 16 mM Na_2HPO_4, 1.3 mM KH_2PO_4, 136.9 mM NaCl, pH=6.75 (53)) and incubated at 37ºC with shaking at 150 rpm. Half of the supernatant was collected at predetermined intervals and replaced with fresh SS. Supernatants were stored at 4°C until analysis.

Release studies were performed in non-sink conditions with volumes comparable to those used for epithelial raft experiments (described separately). Samples of 10 mm diameter were placed on nylon cell strainers (100 µm pore size), which were laid on top of the wells of 24-well plates. Wells were filled with 3.5 mL of SS such that the liquid contacted just the bottom of the films/strainers. Supernatants of volume 0.5 mL were collected at predetermined intervals and were replaced with fresh SS.

Cumulative release profiles were analyzed using the Korsmeyer-Peppas mathematical model (45):

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

where M_t / M_∞ is the drug fraction released at time t, k is a constant depending on structural and geometric characteristics of the system, and n is the diffusional coefficient related to release mechanism. While $n=0.45$ indicates Fickian diffusion, $0.45<n<0.89$ indicates non-Fickian diffusion, and $n>0.89$ indicates case-2 relaxation.

5.2.3 Effect of films on viability of tissues

Because subsequent experiments would involve adhering the mucoadhesive films to epithelial tissues, the effect of films on tissue viability was first assessed using ORL-200 cultures (MatTek, Ashland, MA). ORL-200 tissues are multilayered human buccal tissue models with organized basal cells and multiple non-cornified epithelial cell layers grown on filters. Epithelial raft inserts of inner diameter 9 mm were transferred to 6-well plates and maintained at the air-liquid interface by adding 900 µL of assay medium (Figure 5.1). Film samples of diameter 8.75 mm were sterilized by UV exposure for four hours, attached to the epithelial tissue, and incubated at 37°C in a cell culture environment (5% CO_2). Rafts were exposed to films for 6, 12, or 24 hours. A control group without mucoadhesive film was cultured for 24 hours.

For analysis, tissue inserts were blotted dry, transferred to 24-well plates preloaded with 300 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution, and incubated for 3 hours. Tissue inserts were then transferred to fresh 24-well plates, immersed in extractant solution, and incubated for 2 hours at room temperature on an orbital shaker in the dark. The optical density at 650 nm was subtracted from absorbance at 570 nm.

5.2.4 EGF bioactivity

Bioactivity of EGF loaded into films was confirmed by measuring the proliferative effect of release supernatants on BALB/3T3 fibroblasts (ATCC CCL-163). Activity of "fresh", unprocessed EGF was tested by determining its proliferative effect prior to confirming bioactivity of supernatants from EGF-loaded films. Cells were suspended in 0.5 mL of DMEM supplemented with 2.5% calf serum (Hyclone Thermo Scientific, Waltham, MA) and seeded into 24-well plates at 7,500 cells/well. Cells were allowed to adhere overnight, and then EGF was added to wells at concentrations ranging from 19 pg/mL to 40 ng/mL. After 3 days, proliferation was determined by quantifying DNA contents. Hoechst 33258 (Sigma-Aldrich; St. Louis, MO) was added to cell lysates to achieve 100 ng/mL, and fluorescence (excitation =356 nm, emission = 458 nm) was measured after incubation of 10 minutes at 21ºC.

Mucoadhesive films were then loaded with EGF such that diluted release supernatants reached concentrations within the linear range determined for unprocessed growth factor (37-315 pg/mL). This range was used to avoid false positives because of saturation effects at high concentrations. The volume of release supernatant added to the proliferation assay wells was $\leq$2%. The proliferative effect of EGF in release supernatants was compared to control (fresh EGF in medium) at the same concentration.

5.2.5 Wound healing

Although viability assays were performed on ORL 200 tissues containing only epithelial cells, wound healing studies were performed on full thickness ORL 300-FT epithelial raft tissues, because these full thickness tissues are supported by a collagen bed containing fibroblasts that provide growth factors and other biomolecules in an efficient way to the epithelial cells, mimicking *in vivo* conditions.

5.2.5.1 Time course

The time course of wound healing/closure in ORL-300 FT raft cultures was determined before investigating the efficacy of EGF. Wounds of 3 mm diameter were made using a stainless steel biopsy punch (Huot Instruments, Menomonee, WI). Care was taken to remove only the epithelial part of the rafts, leaving the layers of collagen with embedded fibroblasts intact. Tissue inserts were then transferred to 6-well plates and elevated using two washers to accommodate 5 mL of medium, thus maintaining air-liquid culture conditions for extended periods of time (Figure 5.1). Medium was replaced every other day. Wound closure was determined by measuring the unhealed area after collecting tissues at 0, 2, 4, 6, or 8 days.

5.2.5.2 Effect of EGF

The effect of EGF, EGF-loaded films, and blank films on wound healing was investigated at 3 and 5 days, which were chosen from the prior time course study. Tissues were set up and epithelial wounds made as previously described. A total of 38 tissues was divided into six groups to elucidate the effect of polymers on migration of cells during healing and limiting transport of EGF through tissues: 1) control/EGF-free medium; 2) 5 ng/mL EGF in medium; 3) blank (no EGF) films; 4) EGF-loaded films; and 5) EGF-loaded films placed in medium without contacting tissue. Published *in vitro* and *in vivo* studies showed that 1-5 ng/ml EGF results in maximal cell migration and wound healing in animals (93, 96, 99). Based on the previously determined release profiles, each EGF film was loaded with 300 ng to achieve 5 ng/mL in the medium at the end of 8 hours (removal of film).

For groups that involved application of films, sterilized, 5.6 mm diameter films were applied to 9 mm tissues having 3 mm wounds. Films were carefully removed after 8 hours to avoid tissue damage. The tissues were then incubated with 300 µL of PBS for 30 minutes to solubilize residual polymer that could hinder migration of cells. Medium was replaced every day for all groups to maintain uniformity.

5.2.5.3 Reconstruction of wound and determination of wound area

Collected tissues were removed from inserts, fixed overnight in 4% formalin, embedded in paraffin, and sectioned at a thickness of 10 µm. At every 250 µm across the 9 mm diameter of the tissue, a section was mounted onto a slide, stained with hematoxylin and eosin (H&E), and analyzed for wound area. The length of unhealed wound bed was quantified in two ways, where: 1) thickness of the epithelial tissue was less than 50% of that in unwounded tissue and 2) the collagen bed was devoid of epithelial cells. These measurements of unhealed wound bed were plotted in Excel separated at 250 μm increments, the perimeter encompassing the wound outlined, and the unhealed wound area calculated using ImageJ software (Figure 5.2). Histological features of tissues were assessed by an oral pathologist (C.B.F.)

5.2.6 Statistical analysis

All experiments were conducted with a minimum of triplicates and repeated at least once to demonstrate reproducibility of results. The results are expressed as mean ± standard error unless otherwise noted. While unpaired, two-tailed student t-tests were used to compare bioactivity of release supernatants and "fresh" EGF, ANOVA with the Tukey post-hoc test was used for the rest of the comparisons. Results were considered statistically significant if $p<0.05$.

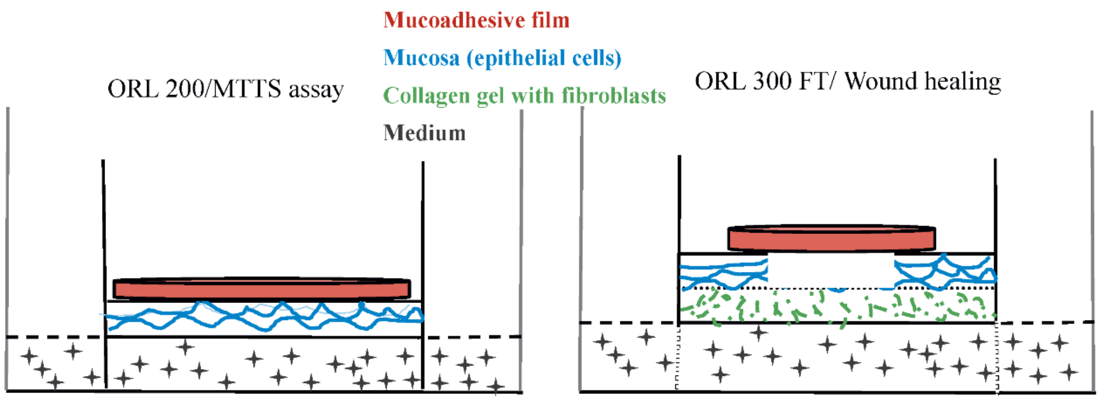


Figure 5.1. Schematic of tissue raft setup and conditions for viability (ORL-200) and wound healing studies (ORL 300-FT). Mucoadhesive films of diameter 8.75 mm were adhered to ORL-200 to cover the tissue surface. Wounds (3 mm) to only the epithelial layer were made in ORL 300-FT using a punch biopsy, and films of diameter 5.6 mm were adhered.

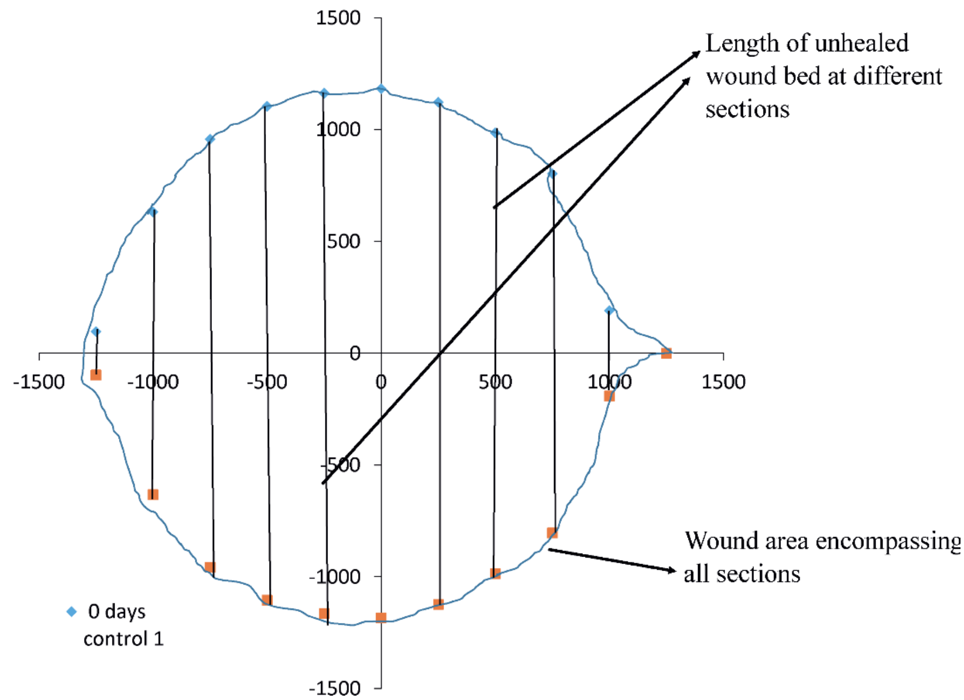


Figure 5.2. Determination of unhealed wound area. The measured widths of unhealed wound were spaced 250 μm apart, and the area encompassing the wound was calculated by ImageJ.

5.3 Results

5.3.1 Release profiles

PVP:CMC mucoadhesive films were able to achieve sustained release of two proteins. Under sink conditions, release of the 14.3 kDa lysozyme was observed for 160 minutes, but the duration was extended up to 320 minutes in non-sink conditions that mimicked those necessary for culture of tissue rafts (Figure 5.3a). Mathematical modeling of the release profiles based on the Korsmeyer-Peppas equation showed 'n' values of 1.30 and 0.715 for sink and non-sink conditions, respectively.

The 1:2 PVP:CMC mucoadhesive films were also able to achieve sustained release of the smaller EGF (6.2 kDa) for up to 360 minutes in non-sink conditions (Figure 5.3b). The desired concentration of 3.5 ng/mL was achieved at 150 minutes and then maintained in the range of 3.5 to 4.0 ng/mL for rest of the release profile. However, the amount of EGF released in 6 hours was observed to be only 58%, unlike 80% of lysozyme released in 320 minutes. EGF was slowly released for the next 18 hours, reaching 88% by 24 hours (Figure 5.4). Applying the Korsmeyer-Peppas model to the release profile for EGF showed an 'n' value of 1.20.

5.3.2 Effect of films on viability of tissues

Viability of ORL-200 tissues decreased 15% compared to control (no films) when films were applied for 24 hr (Figure 5.4). No difference from control was observed at the 6 and 12 hr time points. These results suggest that mucoadhesive films can be applied for up to 12 hr without affecting the viability.

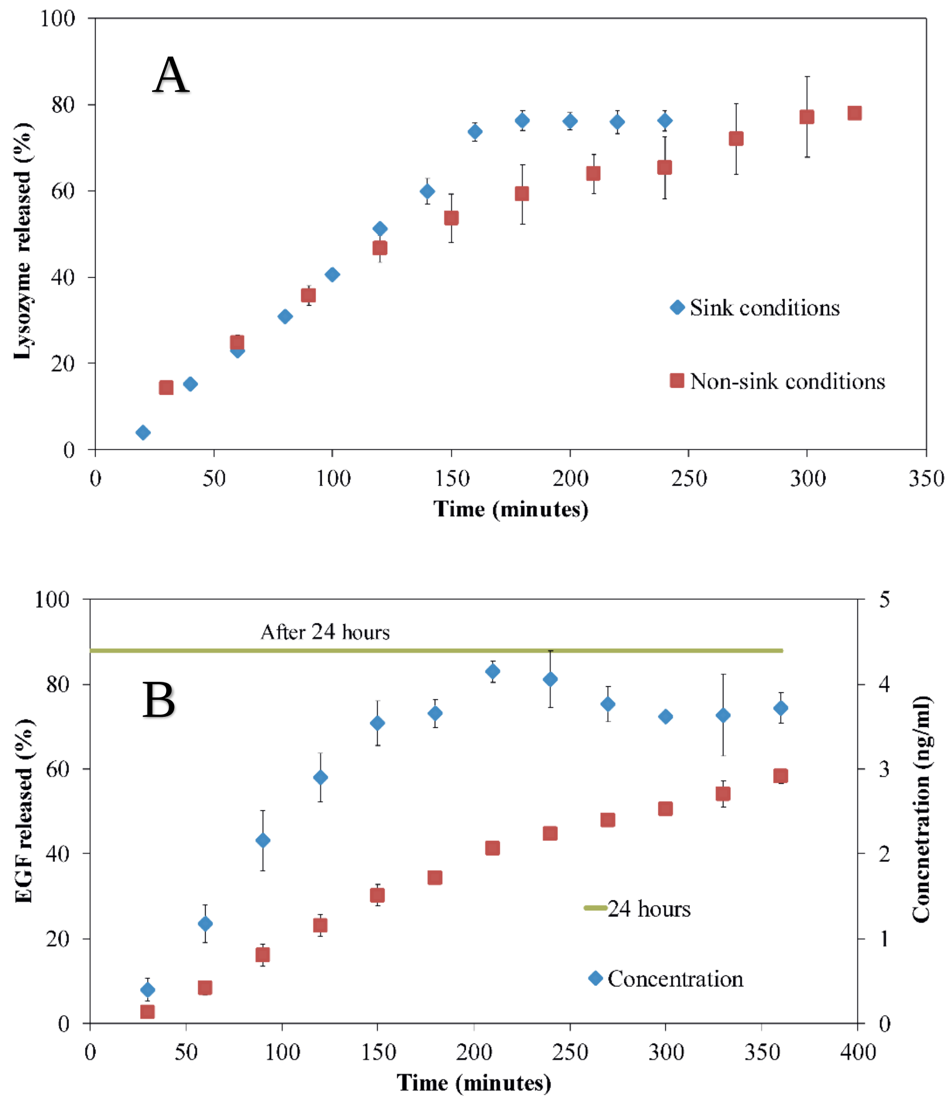


Figure 5.3. A) Cumulative release of lysozyme from 1:2 PVP:CMC mucoadhesive films in sink and non-sink conditions. B) Instantaneous and cumulative release of EGF from 1:2 PVP:CMC mucoadhesive films in non-sink conditions. The horizontal line indicates the cumulative percentage released after 24 hr of incubation. Data are mean ± standard error ($n \geq 3$).

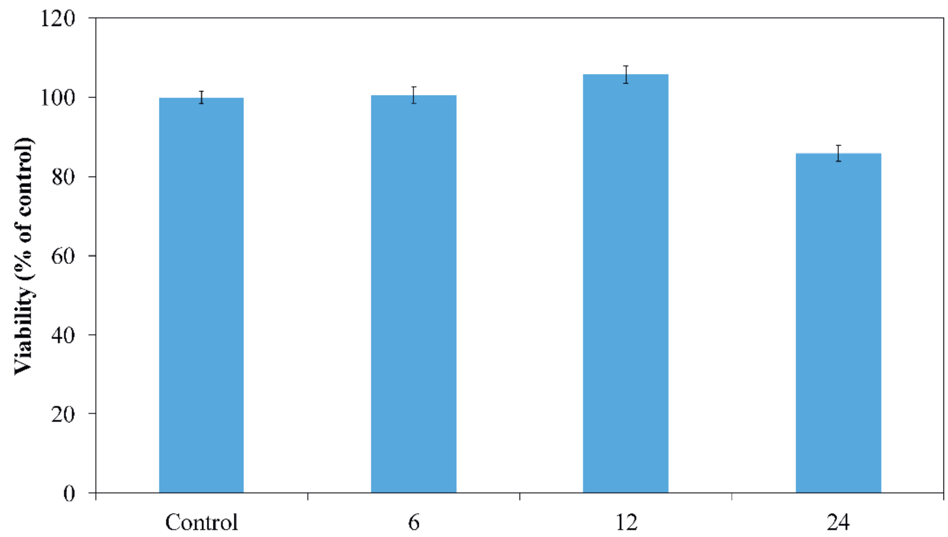


Figure 5.4. Viability of ORL-200 tissues after application of mucoadhesive films for up to 24 hr. Data are mean ± standard error (n≥3).

5.3.3 Bioactivity of EGF

The mitogenic effect of EGF on BALB/3T3 fibroblasts was clearly seen as a biphasic increase in DNA content, which corresponded to the number of cells (Figure 5.5a). EGF significantly ($p<0.05$) increased cell proliferation with increasing concentration up to 315 pg/mL and remained stable from 315 pg/mL to 5 ng/mL, after which DNA contents were lower. The minimum concentration of EGF required to elicit a statistically significant increase in proliferation was 19 pg/mL. The linear range of EGF effect was between 39 and 315 pg/mL. Based on these results, bioactivity of EGF released from mucoadhesive films into SS was tested at concentrations of 75, 150, and 300 pg/mL (Figure 5.5b). The mitogenic effect of EGF released from mucoadhesive films was comparable to that for EGF directly added to media; no significant differences in DNA content were observed between release supernatants and the fresh EGF at the three concentrations tested.

5.3.4 Wound healing studies

5.3.4.1 Time course

A clear time-dependence in wound healing and closure was observed in ORL 300-FT tissues *in vitro* (Figure 5.6). Wound area is shown in terms of control wounds that were created and fixed immediately before healing could occur. The length of unhealed wound in each section was considering unhealed if the epithelial layer was less than 50% of normal thickness. The unhealed wound area was observed to be 60, 30, and 17% by the end of days 2, 4, and 6, respectively, with complete wound closure observed at 8 days. Although one sample in each of the 4 and 6 day groups also healed completely, all other specimens in those groups had a notable amount of unhealed wound area. The time points of 3 and 5 days were chosen as the middle and endpoints to investigate the effect of EGF on healing.

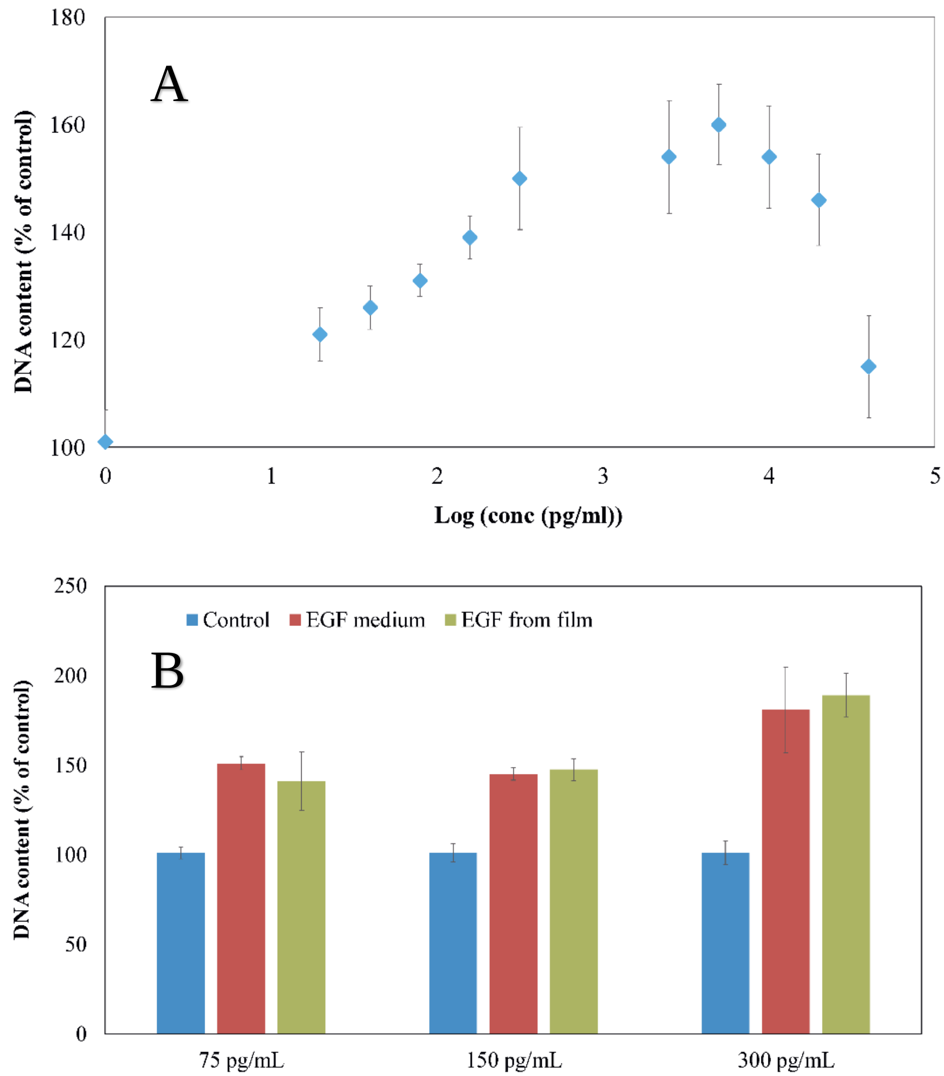


Figure 5.5. A) Mitogenic effect of unprocessed EGF on proliferation of BALB/3T3 fibroblasts. B) Comparison of cell proliferation in response to EGF released from mucoadhesive films with that for unprocessed EGF at three different concentrations. Proliferation was measured by DNA contents and is expressed as a percentage of control (no EGF) cultures. Data are mean ± standard error (n≥3).

5.3.4.2 Effect of EGF films

Because the patterns and absolute values of healing measured by the two methods were different, results are presented for both approaches.

Thickness: Introduction of EGF by any type of delivery (medium, EGF-loaded films adjacent to tissue, and EGF-loaded films directly on the tissue) resulted in decreased wound healing at day 3 (Figure 5.7a), although no significant differences in wound area were observed between groups. Blank films also resulted in decreased wound healing compared to controls, whose wounds were 85% healed. Wounds continued to close from day 3 to day 5, except for the soluble EGF group, which showed a significant ($p<0.01$) increase in unhealed wound area compared to all other groups; no significant differences were observed between the remaining groups.

Epithelialization: EGF did not improve wound healing compared to controls, with no significant difference between groups (Figure 5.7b). However, quantification by epithelialization showed increased healing of wounds compared to the previous thickness method. This increase was more prominent in the EGF-treated groups than non-treated groups. Based on epithelialization of wounds, at day 3, healed wound area increased by 17-27% in all EGF-treated groups and increased by only 5.5 and 4.6% in the non-treated groups. At 5 days, wounds in the controls and tissues treated with EGF films were observed to heal completely, with 90% closure observed in the remaining groups, although the differences were not statistically significant. The increase in healed wound areas due to quantification method remained the same (17-20% in EGF-treated groups, 74% for EGF in medium, and 6 and 9% for the control and blank groups, respectively).

Histological observations of tissues revealed a hyperparakeratotic response in groups treated with EGF, which was not evident in control and blank film groups (Figure 5.8). EGF-treated groups were observed to have other distinguishable histological features, such as acanthosis (thickening of the spinous layer), intercellular edema (clear cytoplasm), intracellular edema (spongiosis), pyknotic nuclei, and a few areas of dyskeratosis (Figure 5.9). The wound bed of EGF-treated groups was observed to be covered with basal cells comparable to non-EGF groups, however spinous and squamous cell layers were not developing. The non-wounded area of EGF-treated tissues, meanwhile, became swollen, and excessive parakeratin was produced (Figure 5.10). After producing keratin, cell layers were observed to flake along with keratin during histological processing. The hyperparakeratotic response was more predominant in tissues treated with soluble EGF and EGF-loaded films adjacent to the rafts compared to those directly exposed to EGF films (Figure 5.10), which was also supported by quantitative analysis (Figure 5.7b).

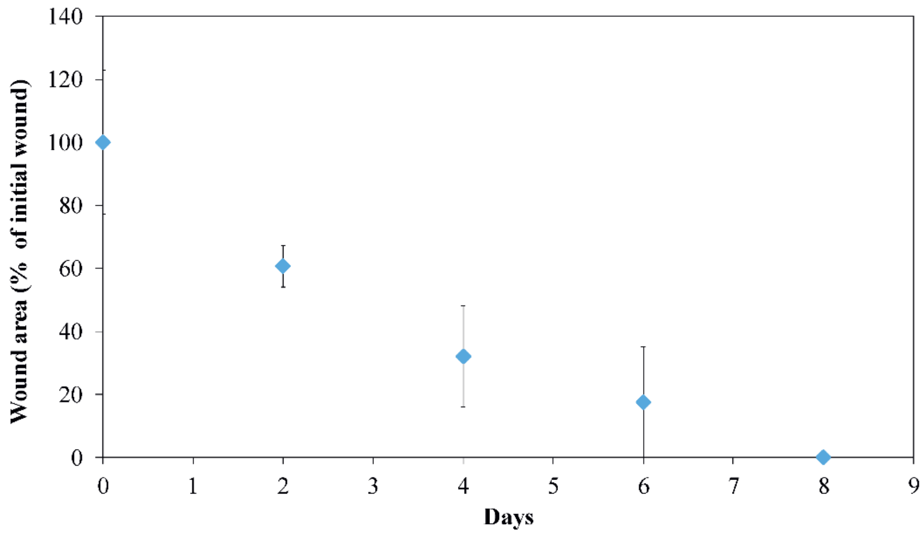


Figure 5.6. Time course of ORL-300 FT tissue healing presented as a percentage of the initial wound (3 mm diameter). Data are mean ± standard error (n≥3).

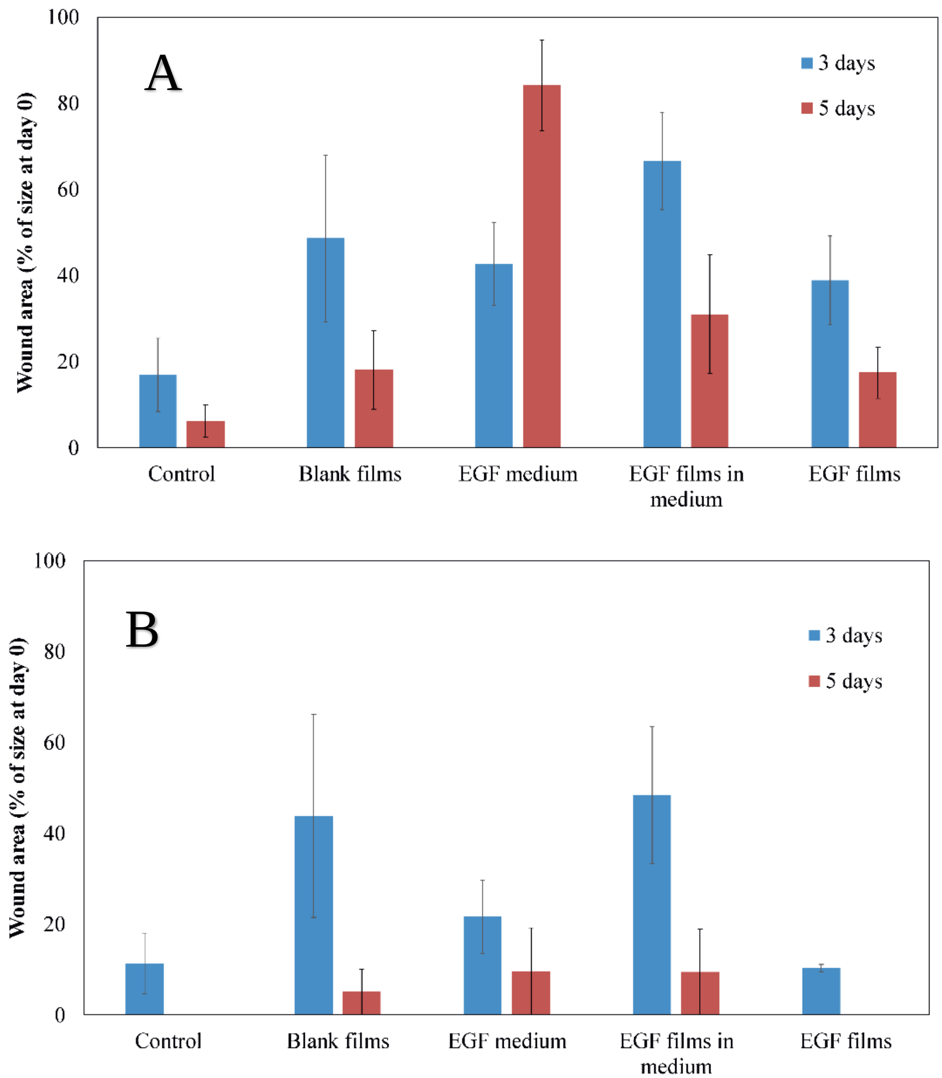


Figure 5.7. Quantification of unhealed wound area based on A) 50% thickness and B) wound bed devoid of epithelial cells. Data are mean ± standard error (n≥3).

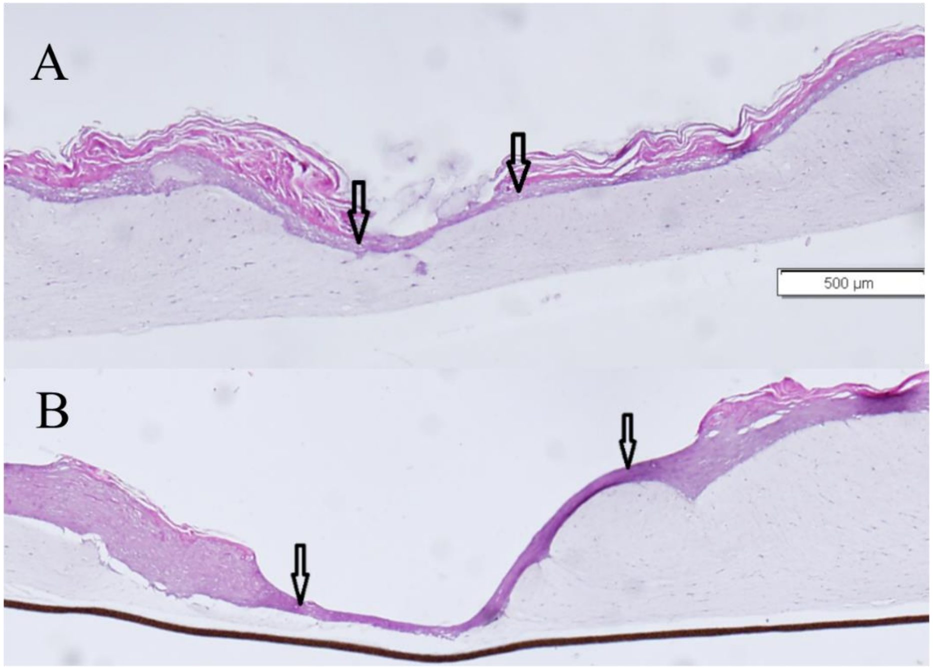


Figure 5.8. Hyperparakeratotic response evident in tissues treated with EGF films compared after 5 days. A) After treatment with EGF-loaded films, excess parakeratin was observed to be stained dark pink and appeared as fibers on the surface of the tissue. B) Less or no parakeratin was observed in control tissues. Arrows represent wound edges

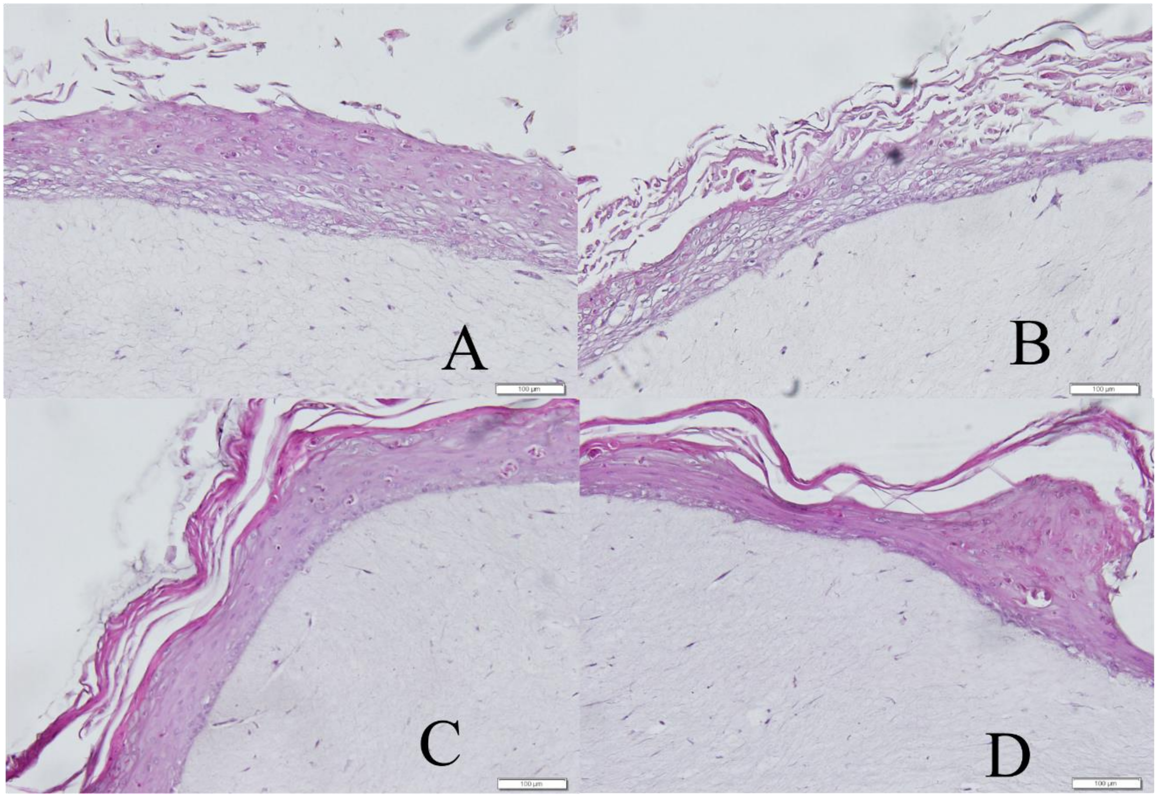


Figure 5.9. Comparison of histological features of EGF-treated and untreated tissues. Moderate and prominent appearance of acanthosis, edema (intercellular and intracellular), and pkynotic nuclei were observed for A) EGF films in medium and B) EGF medium, whereas intact structures were seen for C) control tissues exposed to neither mucoadhesive film nor EGF and D) those with blank films.

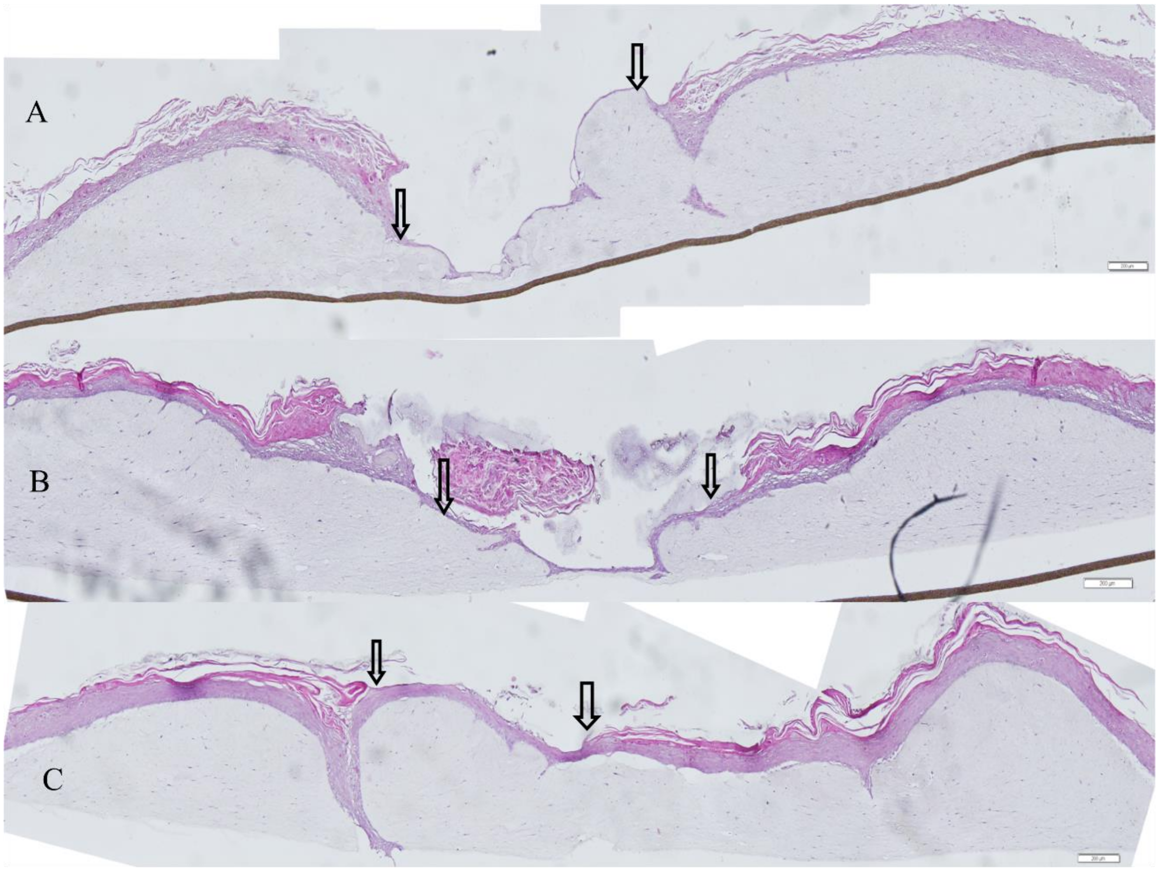


Figure 5.10. Treatment with EGF caused incomplete wound healing. Wound beds were covered with only basal cells in tissues exposed to A) EGF medium or B) EGF films compared to completely healed wounds containing basal cells, a spinous layer, and squamous cell layers for tissues with C) blank films. Arrows represent approximate edges of the wound.

5.4 Discussion

Prominent treatments for oral mucosal deficits include: a) the gold standard of autologous grafts, b) allografts, and c) alternative tissue engineered grafts. In addition to other limitations of autologous grafts, harvesting of mucosal tissue from a donor site can result in several morbidity issues, such as numbness, tightness of the mouth, motor deficits, and contractures (33). Although engineered tissues, such as EVPOME (*ex vivo* produced oral mucosa equivalent) (35), have shown some promising results, use of these grafts must be planned long before surgery. Extraction of a patient's own cells, multiplication of these cells *in vitro*, and finally introduction of these cells onto collagen scaffolds to form 3D grafts typically takes around 8 weeks (35, 91, 100), thus hindering off-the-shelf availability. In addition, viability of the developed grafts, such as EpiOral, is up to only 2 weeks. The present mucoadhesive films are hence being developed to bridge this gap in cases of sudden traumatic injuries to provide protection and aid in regeneration of native mucosa by delivering bioactive molecules. These films may also avoid the need for grafts if significant progress in healing is achieved.

EGF has been shown to promote epidermal wound healing in several animal and a few clinical case studies (92, 101). In addition, EGF plays an important role in mucosal regeneration. This was demonstrated by treating animals unable to produce EGF (sialoadenectomized and diabetic mice) with exogenous EGF (95, 102). However, few studies investigating the use of EGF-loaded devices to treat mucosal wounds are found in the literature. Research performed by Gonul et al. showed efficacy of EGF-loaded poly(ethylene glycol) pellets along with titanium rings in oral mucosal incisional wounds (103). Because the EGF pellets were sutured under tissue, they were protected from the oral environment, and thus the situation is different from treatment of large mucosal wounds. Application of exogenous EGF in methylcellulose gel did not enhance wound healing of 2 mm mandibular alveolar mucosa in healthy rats (104). Application of such systems to wounds may be practically difficult in animal models because they may be removed or compromised by the animals. Hence, an *in vitro* mucosal tissue, *i.e.*, EpiOral (ORL 300-FT), was used to investigate the efficacy of EGF-loaded mucoadhesive films in promoting wound healing.

The present release studies also demonstrated the versatility of the mucoadhesive system in providing sustained release of hydrophilic proteins ranging from 6-14 kDa in addition to small hydrophobic molecules, such as imiquimod (0.24 kDa), shown in previous work (98). The difference in cumulative amounts of EGF (58%) and lysozyme (80%) by the end of 6 hours may be attributed to the concentration gradient arising from the different initial loadings of EGF and lysozyme, *i.e.*, 105 and 4,000 ng, respectively. The observed biphasic effect of EGF on proliferation of fibroblasts is consistent with previous articles of decreased mitogenicity when concentrations exceeded 10 ng/ml (93, 99).

Tissues treated with EGF in any form exhibited a hyperparakeratotic response. Microscopic characteristics observed in EGF-treated groups, such as parakeratosis, acanthosis, pyknotic nuclei and few instances of dyskeratosis, are seen as features of leukoplakia (105, 106). The wound beds of EGF-treated groups were covered with a single basal layer of cells, although with some delay compared to controls. This observation suggests that EGF elicited a different kind of response, driving cells to produce more keratin and edema rather than increasing proliferation to cover the wound. Even though basal cells covered the wound beds, further maturation and differentiation to form spinous layers may have been inhibited by the action of EGF. This response was more evident in groups containing fresh, soluble EGF in the medium compared to those with sustained, unidirectional, and slow release of EGF from films.

Different instances of a hyperparakerotic response of epithelial cells when treated with EGF *in vitro* are evident in the literature. Makarova et al. showed moderate upregulation of cytokeratin 13 (responsible for parakeratin) with high levels of EGF in various head and neck squamous cancer cell lines grown *in vitro* (107). EGF receptor (EGF-R) was upregulated in regions of oral leukoplakia, showing a role for EGF in the histological appearances of acanthosis and pyknotic nuclei (108, 109). Kondo et al. observed that addition of EGF to organ cultures of rabbit ear skin explants caused the epidermis to become acanthotic with orthokeratosis, and further increases in the concentrations of EGF caused parakeratosis (110). Schneider et al. reported that EGF-loaded self-assembling peptide nanofibers caused a five-fold increase in wound healing of *in vitro* human skin equivalents at two days compared to untreated groups (111). The amount of EGF loaded, however, was 1,000 ng compared to 25 ng in the present studies, and because results were shown after only 48 hours, complete closure of wounds did not occur. EGF-treated groups showed epithelial tongues, which were similar to the thin basal cell layers observed in the present study. Histological images of EGF-treated tissues of Scheider et al. also showed dark pink fibers compared to non-treated groups (111).

EGF was shown to be prominent in healing of skin and mucosal wounds in animals, but it must be noted that the current experiments were conducted *in vitro* on multilayered buccal tissues. These rafts are similar to native tissue with multiple epithelial cell layers, collagen loaded with fibroblast mimicking connective tissue, and capable of wound healing (as observed in the control specimens). But several other molecules and proteins that play key roles in controlling EGF-stimulated proliferation and timely differentiation may be missing, causing different response such as hyperparakeratosis. Investigation of EGF-loaded films in animals is required to verify the hyperparakeratotic effect of EGF. Testing of the films in animals can also lead to better understanding of the potential for *in vitro* tissues to mimic/replace small animals in oral mucosal wound healing studies.

5.5 Conclusion

Mucoadhesive films having the ability to deliver bioactive molecules in a sustained manner and adhere to oral mucosal tissues for up to four hours were developed. Although released EGF did not accelerate wound healing of oral tissue rafts, it elicited a hyperparakeratotic response. *In vitro* buccal tissues may not be appropriate for testing the effects of EGF in wound healing without incorporation of other biochemical factors.

Chapter 6 Local Delivery of Imiquimod using Mucoadhesive Films in Hamsters and Residence Time in Humans

6.1 Introduction

Oral squamous cell carcinoma (OSCC) refers to any malignant cancer that arises from squamous epithelial cells in the oral cavity. This is the 10th most common type of cancer and was estimated to affect 42,440 new patients and cause 8,390 deaths in the United States of America in 2014 (21). OSCC is commonly preceded by discolored (red or white) precancerous lesions characterized by abnormal growth (hyperplasia) and maturation (dysplasia) of epithelial cells. The likelihood of progressing to carcinoma depends on the severity of dysplasia. Early diagnosis and treatment of these oral dysplastic lesions can prevent them from progressing to OSCC and avoid further complications (5, 6).

Available treatment options for OSCC, such as radiation and chemotherapy, are used after dysplastic lesions have already progressed to OSCC, and they commonly lead to post-treatment morbidity. Although surgical resection can be performed to excise moderate to severe dysplastic lesions, the procedure results in loss of tissue and compromise of function. Hence, mucoadhesive films loaded with the immune response modifier imiquimod were designed in previous studies for preemptive, noninvasive, and localized treatment of oral dysplastic lesions (98). Mucoadhesive drug delivery systems have been developed to localize the drug at mucosal surfaces, which avoids loss by first pass metabolism and side effects associated with systemic delivery. In addition to increased bioavailability, adherence between the mucoadhesive polymers and absorbing tissue provides high flux and prolonged residence time of the drug at the desired site (112). Imiquimod as Aldara® cream (5% imiquimod) was approved for treating superficial basal carcinoma. Off-label use has shown effectiveness in treating neoplasms of the vulvar epithelium, and the potential for application to melanoma of the intraepithelial oral mucosa and to oral leukoplakia has been reported based on uncontrolled single case studies (7, 8, 47, 50). Because retention of hydrophobic creams such as Aldara® can be compromised in the oral cavity due to the moist tissue surfaces and continuous saliva turnover, a better delivery system developed for intraoral applications may improve the local and sustained release of drug.

The previously developed mucoadhesive films were able to achieve sustained release of imiquimod for up to 3 hours *in vitro* (75, 98). The *ex vivo* residence time, transport kinetics, and bioactivity of imiquimod-loaded films were also characterized as a function of composition. Although commonly used for initial screening of formulations, the *in vitro* behavior of a device will not necessarily reflect the performance *in vivo* because of differences in clearance rate, mechanical loading, pH, biochemical activities, *etc*. Consequently, *in vivo* testing is required to determine the actual residence time, release kinetics, and ability of the system to deliver drug to tissue. Relatively few reports describe testing mucoadhesive films in animals, particularly those with application sites that enable prolonged residence (104, 113). The hamster cheek pouch model, which remains the most widely used for OSCC studies may provide inaccessible regions of buccal mucosa with physiological similarities to human tissue (26, 114).

The focus of the present studies was to conduct a preclinical evaluation of the performance characteristics of a mucoadhesive delivery system *in vivo*. After evaluating the residence time of films at different application sites in the hamster cheek pouch, the ability of the films to deliver and retain imiquimod in the oral mucosa with minimal systemic distribution was determined. Subsequently, the residence time of drug-free films at different intraoral sites was determined in human subjects.

6.2 Materials and Methods

6.2.1 Materials

Imiquimod was purchased from CalBiochem (White House Station, NJ). The polymers used for making films were polyvinylpyrrolidone (PVP) K-90 (Spectrum; New Brunswick, NJ) and carboxymethylcellulose (CMC; sodium salt, medium viscosity; Sigma, St. Louis, MO). Other chemicals used were propylene glycol, USP grade ethanol (190 proof), methanol, poly(ethylene-co-vinyl acetate) (PEVA; 18wt% vinyl acetate; Sigma-Aldrich, St. Louis, MO); solid phase extraction tubes (STRATA XC; Phenomenex, Torrance, CA) and a generic version of Aldara®, 5% imiquimod (Perrigo, Dublin, Ireland)

6.2.2 Fabrication of films

Bilayered, imiquimod-loaded mucoadhesive films were fabricated as described previously (98). Briefly, aqueous solutions of PVP (1:1:1 ratio of 40% w/v in deionized water: ethanol: propylene glycol) and CMC (2% w/v) were added to imiquimod solubilized in 3:7 acetate buffer (100mM, pH 4):methanol, mixed thoroughly, and stored overnight at 43 ºC to remove bubbles. The backing layer was prepared by casting 10% w/v PEVA in toluene into Teflon dishes and drying at 30 ºC for 48 hours in sealed containers (98). Bilayered films were subsequently made by casting the mucoadhesive polymer solution onto the PEVA and drying at 60 ºC. Films were then removed from the dishes and stored in a desiccator at -10 ºC. Mucoadhesive films with two compositions (1:2 and 2:1 PVP:CMC) and two different thicknesses were prepared (Table 6.1).

Blank films were similarly fabricated for human studies by mixing a drug-free solution of acetate buffer:methanol into the polymer solutions. Samples of diameter 7 and 10 mm were then punched and used for the hamster and human experiments, respectively. All films samples were terminally sterilized by exposure to UV light for 1.5 hours on each side in addition to aseptic fabrication using sterile solvents.

Table 6.1. Thickness of the PVP:CMC mucoadhesive films tested.

Type (PVP:CMC)	Thickness (mm)
1:2 thin	0.36±0.032
1:2 thick	0.55±0.026
2:1 thin	0.36±0.018
2:1 thick	0.47±0.008

6.2.3 Residence time and distribution of imiquimod in hamsters

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). Forty four male golden Syrian hamsters weighing 90-115 g (Harlan, Indianapolis, IN) were used. With animals under mild isoflurane anesthesia, mucoadhesive films were applied to the left cheek pouch by gentle pressure for 10 sec. Hamsters were then transferred to an empty plastic cage with no bedding, food, or water for the remainder of the experiment. Blood was collected for drug measurement by cardiac puncture under deep anesthesia, and the animals were immediately euthanized by CO_2 asphyxiation. The treated and control (right side) cheek pouches were excised and assayed for drug content.

6.2.3.1 Pilot study

A small pilot study was initially conducted to identify the preferred films and application site for further analysis of drug distribution. The four types of mucoadhesive films shown in Table 6.1 were loaded with either a low or high dose of imiquimod, i.e., 0.675 mg/cm^2 (266 µg/sample) or 1.25mg/cm^2 (533 µg/sample). After application of the films, cheek pouches were visually examined at intervals of 2 hours under mild anesthesia. The time point at which either the backing layer was spit out by the animal or the film was absent during visual examination was recorded as the residence time. Residence was investigated at three different locations: cheek, middle of the pouch, and deep within the pouch. After determining the best location to apply films, all four types at both the low and high dose were applied to only the middle of the pouch to assess drug delivery.

6.2.3.2 Local retention of imiquimod

Based on results of the pilot studies, thick 1:2 PVP:CMC films were selected to investigate the local retention of imiquimod in mucosal tissue. A total of 36 hamsters were divided into three groups to test 1:2 PVP:CMC-high dose (1.25 mg/cm^2), 1:2 PVP:CMC-low dose (0.675 mg/cm^2), and 5% imiquimod cream. For the generic version of Aldara®, 5.0 mg of cream (266 µg of imiquimod) were applied to 0.384 cm^2 area of tissue to achieve the recommended concentration of 0.675 mg/cm^2. Animals were euthanized at 2, 4, 8, and 12 hours after application of the film/cream.

6.2.4 Quantification of imiquimod

Imiquimod was first extracted from 0.5 mL blood samples by solid phase extraction (SPE) using Strata-XC columns. Briefly, whole blood was diluted with 3 parts of acetonitrile containing 1% formic acid and centrifuged at 2000 g for 10 minutes. The resultant supernatant was collected and diluted with an equal amount of deionized water. This solution was then loaded in to pre-conditioned SPE tubes to remove impurities, such as phospholipids. Imiquimod was then eluted into 5% ammonium hydroxide in methanol for further analysis.

Tissues excised from hamsters were immediately transferred to 10 mL of acetate buffer (pH 4.0, 100mM) and incubated at 37ºC overnight with shaking at 150 rpm to extract imiquimod (98). The resulting solution was filtered (0.2 µm) to remove tissue particles before further analysis.

The amount of imiquimod extracted from blood and tissues was determined by reverse phase high performance liquid chromatography (HPLC) using a Hitachi Primaide system equipped with a Kinetex C_{18} column. Imiquimod was separated from other constituents using a gradient mobile phase ranging from 20:80 to 80:20 acetonitrile to water containing 0.1% TFA at a flow rate of 1 mL/min. Imiquimod was detected at a wavelength of 242 nm.

6.2.5 Residence time in humans

Under a study protocol approved by medical Institutional Review Board (IRB) of the University of Kentucky, the residence time of all four types of mucoadhesive films (Table 6.1) was also determined in human subjects (age: 22-50, 10 male and 2 female). Film samples of 10 mm diameter were applied to three different locations (left buccal mucosa, dorsal side of tongue, and anterior mandibular gingiva) with gentle finger pressure for 10 seconds. Subjects were asked to record two time points: 1) the time at which s/he felt any change in position of the film from its original location and 2) the time at which the film was completely eroded and/or the PEVA backing layer was completely dislodged from the application site. PEVA backing layers were collected to measure mass and, thereby, to quantify any residual mucoadhesive film. Subjects were asked to refrain from eating and drinking during the course of the study.

6.2.6 Statistics

All experiments were conducted with a minimum of triplicates, meaning that at least three animals or human subjects were used for data collection at each time point. The results are expressed as mean ± standard deviation unless otherwise noted. ANOVA with the Tukey post-hoc test was used to compare groups. Results were considered statistically significant if $p<0.05$

6.3 Results

6.3.1 Application site and pilot study of imiquimod release

The residence time of films varied substantially at different application sites in hamsters. Films applied to the cheek were easily recognized by the animals and removed within 15-35 minutes. The recovered films appeared swollen, with only a small portion of the film eroded. This behavior was observed irrespective of film type and thickness. Films were then tested in cheek pouches accessed by two methods. In the first, the pouch was revealed without complete eversion (Figure 6.1a). This method exposed the outside and inside surfaces of the pouch, shown by yellow and blue labels, respectively. Films applied to the middle region of the inside surface stayed for a variable time, ranging from 3-8 hours, but films applied to outside surface consistently showed a residence time of 3-4 hours. Films were completely eroded in both locations, and the backing layer was recovered from 90% of the animals. In the second method, the cheek pouch was completely everted to access the deepest part of the pouch (Figure 6.1b). When films were applied to this region, the residence time was consistently extended to 8-9 hours. Because the residence time of films applied to the outside surface of the cheek pouch was consistent as well as being close to the anticipated duration in humans, it was selected for further analysis.

The pilot study also showed that the amount of imiquimod extracted from tissue treated with low dose films (0.675 mg/cm^2) was 10 times higher for thicker 1:2 PVP:CMC films and was doubled for thicker 2:1 PVP:CMC films when compared to their thinner counterparts. This trend was also observed for the high dose films (1.25 mg/cm^2). Because of these findings, combined with the better sustained release previously measured *in vitro* (98), thick 1:2 PVP:CMC films were selected for further study of imiquimod retention in mucosal tissue. Macroscopic manifestations of inflammation, such as erythema, erosion, edema, and swelling, were not observed on the tissue after treatment with imiquimod-loaded films through 12 hr.

6.3.2 Local retention of imiquimod

Thick 1:2 PVP:CMC mucoadhesive films delivered imiquimod to the buccal mucosa. The amount of drug retained in the tissue at 2 and 4 hr was maintained at 71 and 50 µg (27 and 20% of drug applied), respectively, for low dose films and 96 and 76 µg (18 and 14% of drug applied), respectively, for high dose films, as long as the films remained adherent to tissue (Figure 6.2). After complete erosion of films by 3-4 hr, drug content decreased significantly ($p<0.05$) to 10-20 µg as observed at the 8 and 12 hr time points. Use of high dose (526 µg vs. 266 µg in low dose) films did not double the local retention of drug, but a significant increase ($p<0.01$) of 35% was measured at 2 hr, with no significant differences observed at the remaining time points. Application of 5% imiquimod cream resulted in 160 µg (60% of amount applied) of drug in mucosal tissue at 2 hr. The time at which the cream was washed away and/or eroded was difficult to measure because of the relatively small amount used and the color of the ointment. The tissue drug content was maintained around 100 µg (38%) for up to 8 hr, after which clearance decreased the amount to 33 µg. Imiquimod retained in the tissue following application of the cream was significantly higher ($p<0.01$) than the amounts for both low and high dose films at only 2 and 8 hr.

6.3.3 Blood concentrations of imiquimod

No traces of imiquimod were found in the blood of hamsters treated with either dose of mucoadhesive film (Figure 6.3). Application of 5% imiquimod cream, however, resulted in imiquimod being found in the blood of 50% of the animals (6 out of 12 hamsters). While one animal each at the 2 and 4 hr time points showed imiquimod concentrations of 8.56 and 248 ng/mL, two animals each at 8 and 12 hr time points had concentrations at an average of 18 and 12 ng/mL, respectively.

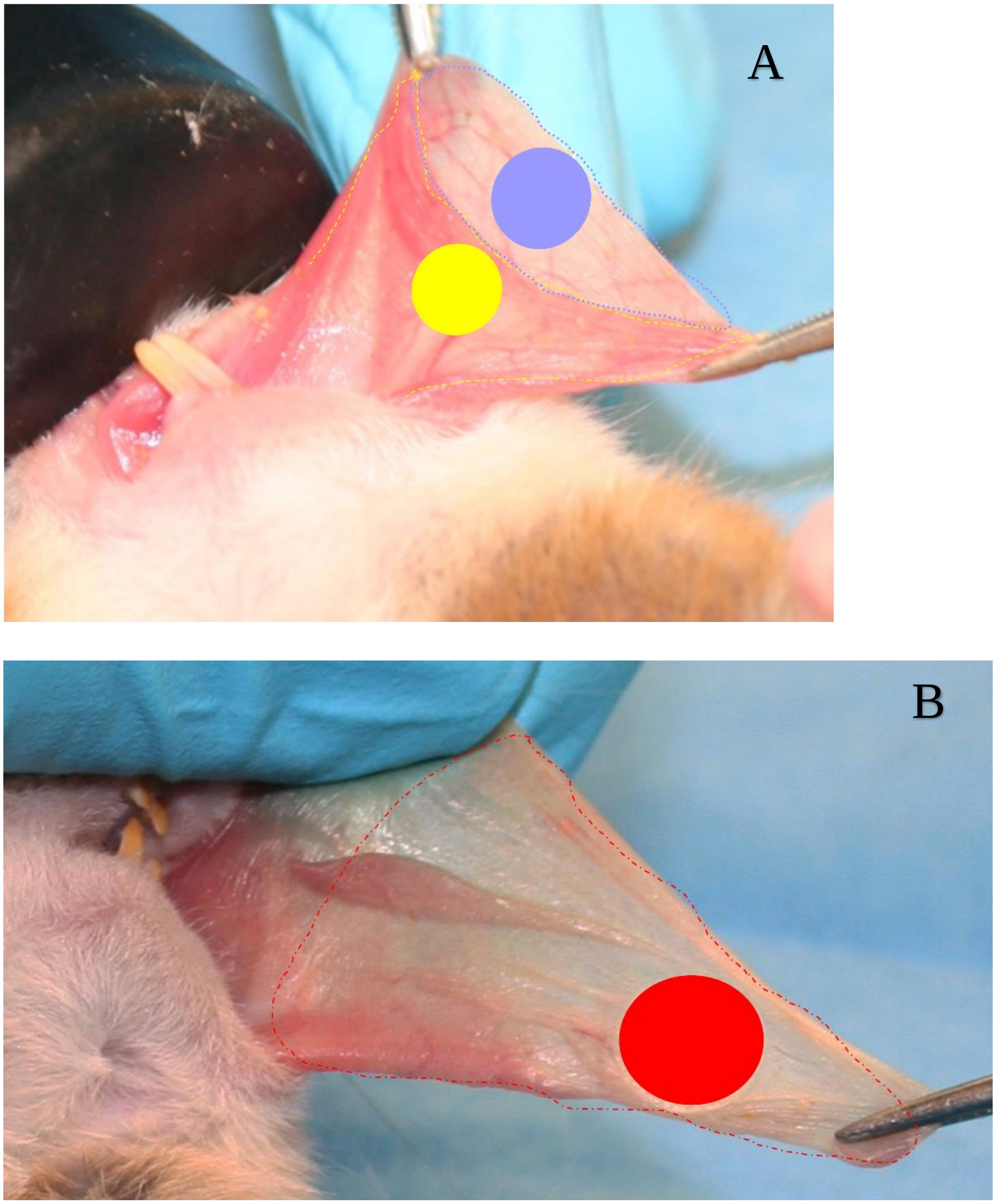


Figure 6.1. Different sites for applying mucoadhesive films in the hamster cheek pouch model. A) The pouch was revealed without eversion, exposing both the outside (yellow) and inside (blue) surfaces. B) The pouch was completely everted to allow access deep into the pouch

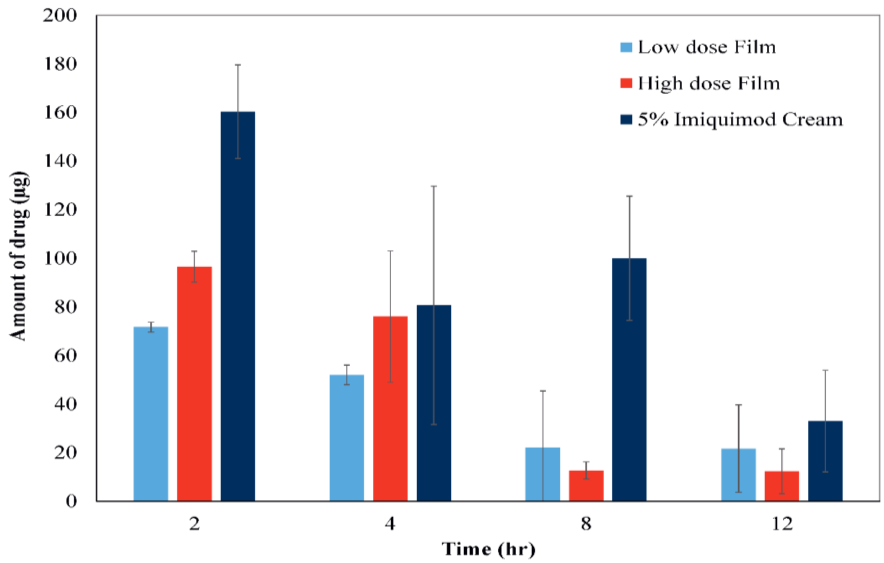


Figure 6.2. Amount of imiquimod retained in hamster mucosal tissue at increasing times after application of thick 1:2 PVP:CMC mucoadhesive films (low or high dose) and 5% imiquimod cream. Data are mean ± standard deviation (n≥3).

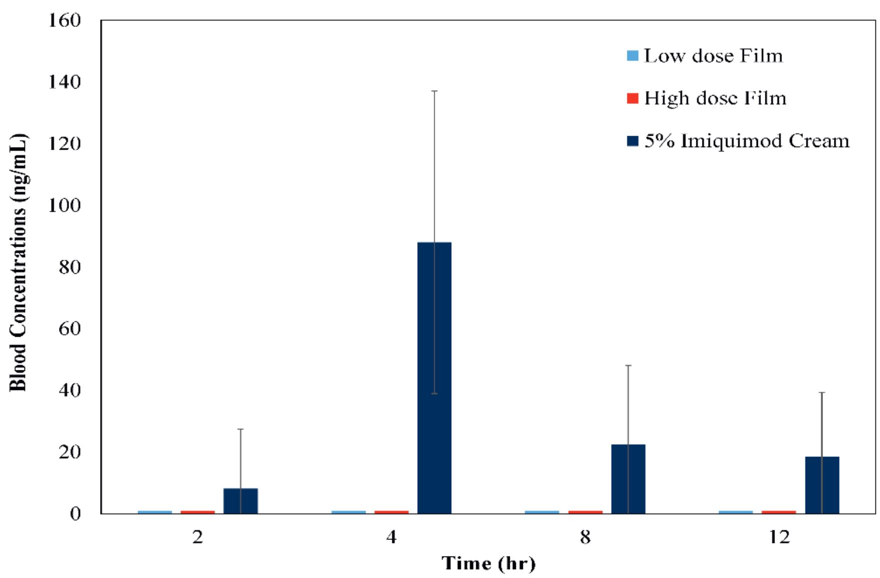


Figure 6.3: Concentrations of imiquimod absorbed into blood of animals at increasing times after application of thick 1:2 PVP:CMC mucoadhesive films (low or high dose) or 5% imiquimod cream. Data are mean ± standard deviation (n≥3). While 266 µg of imiquimod was loaded in each sample of low dose films and cream, high dose films contained 526 µg.

6.3.4 Residence time in humans

Application of mucoadhesive films at different sites in the oral cavity resulted in a range of residence times. Displacement or slight movement of films from their original location was reported at an average of 70% of their final residence time (Figure 6.4A). At cheek and gingiva application sites, thicker 1:2 PVP:CMC films stayed for longest times without initial displacement, but no differences were found on tongue. While no significant difference in displacement times was found between 1:2 thin and thick versions, 1:2 thick films were significantly different ($p<0.05$) from both types of 2:1 at cheek and gingiva.

In conjunction with the above pattern, thicker 1:2 PVP:CMC films exhibited the final longer residence time at the majority of application sites (Figure 6.4B). While films applied to the tongue resided for the shortest time (1.1-1.4 hr), films exhibited the longest residence times on the gingiva (1.4-4.1 hr), depending on the film type. Significant differences between the residence times of different films were not observed on the tongue. Although both the thin and thick 1:2 PVP:CMC films were retained on gingiva for approximately 4 hr, the residence time for only the thin 2:1 PVP:CMC films was significantly lower ($p<0.01$ and $p<0.05$, respectively). The residence time of films on the cheek was intermediate between that for the gingiva and tongue sites. On the cheek, only the thick 2:1 PVP:CMC films were significantly ($p<0.05$) different from thick 1:2 PVP:CMC films.

6.4 Discussion

Imiquimod in the form of a 5% cream (Aldara®) was approved by the FDA for three indications: nonhyperparakeratotic actinic keratoses, superficial basal cell carcinoma, and external genital warts (115). Off-label use of this cream has been for treating mucosal surface disorders, primarily for lesions at different cancerous stages (6, 47, 50, 115). A recent study of Aldara® in hamsters showed reversal of chemically induced well-differentiated OSCC to mild dysplasia (116). Application of this cream in rats also halted progression of OSCC induced by local application of carcinogen and it reversed mild-moderate dysplastic lesions to hyperplastic lesions (117). In clinical trials using Aldara® cream to treat various skin disorders, pharmacokinetic studies showed maximum serum concentrations of 0.1-0.2 ng/mL at first dose, and 1.3-2.2 ng/mL after the last dose, reflecting minimum systemic absorption and localization (115).

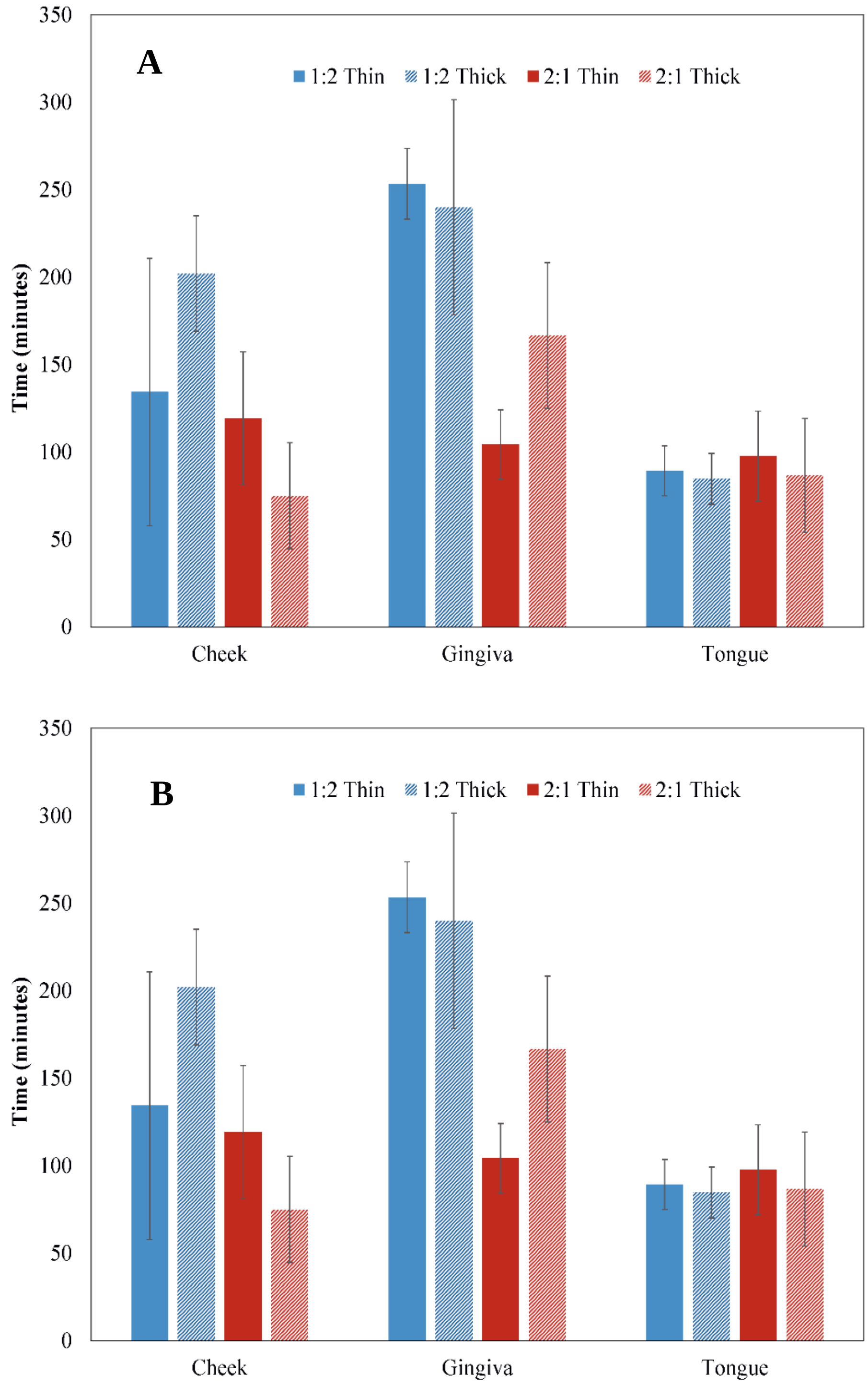


Figure 6.4. Time at which films were slightly displaced from their original location (A) and final residence time of mucoadhesive films (B) at three different application sites in human subjects. Data are mean ± standard deviation (n≥3).

The effect of this localization of imiquimod was clearly shown in the findings of Imbertson et al., in which the concentrations of interferon (IFN) and tumor necrosis factor (TNF) were significantly higher in the treatment area compared to contralateral, untreated sites (118). The amount of imiquimod retained in the treatment area and clearance of the drug have not been reported in either animal studies or clinical trials. Knowledge of local drug contents is useful for designing drug delivery systems. For example, understanding retention of imiquimod in tissues can be helpful in translating treatment from dermal to mucosal indications because the permeability of drugs increases by 4-4000 times in the latter (63). Such information can also provide a basis for adjusting the dosage regimen to achieve therapeutic efficiency and avoid side effects. Hence, local retention of imiquimod in the buccal mucosa of hamsters was quantified and compared between mucoadhesive films and commercially available 5% imiquimod in addition to determination of residence time. Although films were discarded by hamsters within 4 hours, time points of 8 and 12 hours were introduced to measure the clearance rate of imiquimod. Extraction of imiquimod using acetate buffer was established and validated *ex vivo* in porcine mucosal tissues by accounting for the total imiquimod content (98).

Although mucoadhesive formulations, such as Eudispert-Polycarbophil hydrogels and methylcellulose gels, have been investigated for treatment of disorders in a limited number of animal studies (102, 113), key properties such as prolonged residence and release profiles were not reported. Several compounds have been tested either by adding them to water or as liquid and cream formulations (116, 117, 119, 120). The present mucoadhesive films, which were designed to last for 4-6 hours, require a suitable animal model to check the efficacy of treatments in cancer models. Literature searches show common use of mice, rats, hamsters for models of oral disease (121-123). Rodents that are lower on the phylogenetic tree are more useful for mechanistic studies and early-stage testing of potential treatments. Testing mucoadhesives in mice is not practical, however, because of their small size. Even with the larger rats, the small buccal or palatal surfaces are easily accessed by the tongue, increasing the likelihood of film dislodgement. In contrast, placement of mucoadhesives in the buccal pouch of hamsters was anticipated to isolate the films long enough to determine their performance characteristics. The hamster cheek pouch model also resembles human tissue in many respects (26, 114). For example, the hamster cheek pouch shows histological similarity to the human cheek, although the hamster has thinner mucosa than that found in the upper digestive tract of humans. In addition, the hamster OSCC model exhibits several prominent characteristics of human OSCC, including the appearance of transepithelial dysplasia and aberrant expression of cytochrome P450, p53, p21, and Bcl-2 proteins, thus relating well to human disease (26, 124).

While the mucoadhesive films showed erosion times of only 4 hours *in vitro* (75), films placed deep into cheek pouches stayed for 8 hr. This behavior is attributed to the relative dryness deep within the pouch compared to the edges and the rest of the oral cavity. In contrast to consistent residence times (3-4 hr) on the outside surface of the pouch, mucoadhesive films adhered to the inside, middle surface displayed a wide range of residence times (3-8 hr), likely because of differences in the animals accessing the films and pushing them either deeper into the pouch or into the mouth. Films were accessed by all animals before being spit out and were chewed or played with irrespective of application site. Although the residence time of imiquimod cream was not found in hamsters because of practical difficulties, previous *in vitro* studies[10] showed that the cream stayed for a maximum time of only 1 hr.

The residence time of thick 1:2 PVP:CMC films in human subjects (3-4 hr) was similar to that of films applied to the outside, middle surface of the hamster cheek pouch. Other groups reported residence times of 2-8 hr for mucoadhesive films of different polymer compositions in human subjects (15, 125). Such studies, however, were performed on only the upper gingiva instead of different regions of the oral cavity.

Neither the drug-free films nor those loaded with imiquimod stimulated gross inflammatory reactions that would be reflected by erythema and swelling. Other local site reactions, such as itching or burning, were difficult to measure in animals, however hamsters appeared comfortable throughout the experiment. What remains unknown is whether such reactions would appear following repeated application of the films. The human subjects did not report any side effects.

The high tissue content of imiquimod (160 µg) in the cream group may be attributed to the easier penetration of drug into the tissue compared to the slow and sustained release from the mucoadhesive films. In addition, the fatty acids and alcohols used in the formulation, which was developed for application to skin containing hair follicles (65), increase permeation of drug through mucus and the epithelial cell layer (9, 12, 126). Although fatty acids increase solubility and permeability of lipophilic drugs, their extraction and disruption of lipid bilayers and intercellular lipids during this process has been reported to cause irritation and swelling of the buccal mucosa (9). The product information sheet for 5% imiquimod cream designed for treatment of skin disorders also describes itching, burning, and irritation as common side effects (127).

Measurement of imiquimod in the blood of half of the animals treated with cream reflects significant systemic absorption of drug, which was not evident following application of both low and high dose mucoadhesive films. Imiquimod, enhances the innate and acquired immune responses by activating monocytes and macrophages to secrete several cytokines, such as tumor necrosis factor (TNF-α) and interleukins (46, 97). This upsurge of immune response may be responsible for systemic side effects, such as fevers, dizziness, muscle pain, and flu-like symptoms, observed for Aldara® cream (127). Minimal systemic absorption and increased localization of drug will decrease these side effects.

Although mucoadhesive films did not prolong drug retention as long as the cream (up to 8 hr) did, their ability to deliver imiquimod and retain the drug in tissue while the films remained adherent was clearly evident. The role of mucoadhesive polymers in improving the retention of imiquimod was previously shown by PVP:CMC films increasing the localization of imiquimod in porcine buccal mucosa by 50% compared to drug solution.[4] Hence, an increased residence time can further increase bioavailability of drug for 8 to 12 hr at therapeutic doses. Owing to a versatile manufacturing process, these films can also be loaded with different compounds to increase permeability if needed. Further studies would be needed, however, to balance increased permeability with no systemic absorption and tissue irritation. Overall, the cream resulted in poorly controlled delivery, whereas mucoadhesive films moderated imiquimod release to help avoid significant systemic distribution of the drug.

6.5 Conclusion

Mucoadhesive films developed to treat oral dysplastic lesions by sustained release of imiquimod demonstrated a residence time of up to 4 hours in humans and animals. The 1:2 PVP:CMC mucoadhesive films were able to deliver imiquimod to oral mucosal tissue and also help in localization of drug in tissue up to 12 hours with no systemic absorption.

Chapter 7 Summary and Conclusion

A mucoadhesive film capable of delivering imiquimod to mucosal tissues has been developed to treat oral precancerous lesions also known as dysplastic lesions. The developed system offers noninvasive approach of treating precancerous lesions in contrast to current invasive approaches. Problems caused by hydrophobic nature of imiquimod such as maximum drug loading capacity and uniformity in the drug delivery system, were addressed by using acetate buffer as a solvent, after investigating three methods of imiquimod loading during fabrication of the films. The bioactivity of imiquimod was not effected by its entrapment in the polymer matrix and manufacturing steps. The films displayed adequate adhesion time *in vitro* on porcine mucosal tissues and significantly higher than commercially available Aldara® cream.

To further understand the properties of the designed system, the compositions of polymers PVP and CMC were changed to fabricate five types of films. These films are compared with respect to key properties of a mucoadhesive system such as tensile, pull-off and shear adhesion, swelling, erosion characteristics and release profiles. The mucoadhesive system developed in this dissertation offered wide range of all these properties, and 1:2 PVP:CMC type of film was selected for further studies. Sustained release of imiquimod was achieved for 3 hours *in vitro* and release of imiquimod is controlled by erosion of the films. The impermeable and non-degradable backing layer facilitated release of imiquimod only to mucosal tissue and avoided loss of drug through the oral route.

The films demonstrated a residence time of up to 4 hours *in vivo* in humans and animals, comparable to *in vitro* findings. In addition to providing controlled release, the mucoadhesive polymers also nearly doubled the retention of the imiquimod in epithelial tissue of porcine mucosa *in vitro*. The retention of imiquimod was also observed *in vivo* in hamster cheek pouches. The films delivered imiquimod to oral mucosal tissue and helped localize the drug while they remained adherent for 4 hours, after which tissue drug content decreased through 12 hours with no systemic absorption of imiquimod in to blood. Although commercially available imiquimod cream resulted in higher drug contents through 8 hours, half of the animals showed systemic absorption of imiquimod. Because the films intend to provide localized treatment of dysplastic lesions with minimal side effects of the drug, zero systemic absorption and localization effect observed *in vivo* increases the potential of translation of films in to clinic.

In addition, the versatility of films to load with different active molecules such as proteins was proven by fabricating EGF loaded films for the treatment of mucosal wounds. The mucoadhesive films also showed similar sustained release of lysozyme and bioactive EGF as observed with imiquimod. When the efficacy of EGF loaded films in promoting wound healing, was tested on *in vitro* buccal tissues, they elicited a hyperparakeratotic response causing significant morphological changes. Although complete wound healing was not promoted in this work, with the potential for sustained release of bioactive growth factors and small molecules, the developed mucoadhesive delivery system may be loaded with other desired compounds in conjunction with or without EGF to accelerate the process of wound healing.

Overall, a mucoadhesive system capable of delivering bioactive small molecules and proteins in sustained manner was developed in this work. A thorough understanding of the system properties was achieved to further tune for future applications. *In vitro* studies and *in vivo* studies in hamsters and humans clearly showed the potential and usefulness of the system to translate in to clinic for the treatment of oral precancerous lesions. Further improvement of residence time by modifying polymers in future studies, can significantly enhance the potential of current system in treating several other disorders.

APPENDIX

A.1 Introduction

Mucoadhesive films developed in this dissertation exhibited maximum residence time of only 4-5 hours, as observed in *in vitro* and *in vivo* studies in earlier chapters. Prolonged adherence of the films to the oral tissue can further extend the delivery of imiquimod for more than 4 hours. In addition, long residence time of the films can be advantageous for other local disorders such as oral tissue regeneration and mucositis. Hence series of experiments were conducted by either incorporating additional new polymer or change properties of current polymers, without significant changes in the original composition of polymers. The objective of work in this section was to improve all the essential properties such as erosion time, mucoadhesion time, and extended drug release.

A.2 METHODS

Common methods used to analyze different film types in this chapter are described below in this section.

A.2.1 Fabrication of films:

Films made up of 1:2 PVP:CMC composition were selected for further study of increasing the residence time and erosion time. These films were renamed as 80:20 PVP:CMC composition in this appendix for easy comparison with other film types. Either new polymers or existing polymers were added/substituted to former composition during fabrication of different types of films in this appendix. Fabrication process of films remained same as described previously in chapter 3. After aqueous polymer solutions of PVP and CMC were mixed, new polymer solution (where applicable) were added to them along with drug solution and thoroughly mixed, stored at 43^o C overnight, and cast dried in Teflon dishes at 60^o C.

A.2.2 Swelling studies

Film samples of diameter 10 mm, with PEVA backing layer were incubated in 6 mL of SS at 37ºC. The dry weight (W_1) was recorded before immersion. Samples were then removed at predetermined intervals, blotted dry from the backing layer side, and the weights were recorded as W_2. Films were compared based on the swelling index, which was calculated as $[(W_2-W_1)/W_1]*100$.

A.2.3 *Ex vivo* mucoadhesion time

Film samples of diameter 10 mm with PEVA backing layer were attached to the mucosal surface of pre-hydrated (50 µL SS) porcine buccal tissue with slight finger pressure. Tissue samples were attached to a glass slide with cyanoacrylate glue. The glass slide was fixed to the moving actuator of a BOSE ELF3300 mechanical testing system and allowed to move up and down into 700 mL of SS at a rate of 18 cycles per min. The film was completely immersed in the buffer solution at the lowest point and was out of the solution at the highest point. The time at which partially eroded films or the backing layer of completely eroded films was detached from the tissue was recorded as the *ex vivo* mucoadhesion time.

A.2.4 Release and Erosion studies

Samples of diameter 10 mm with PEVA backing layer were punched from random points of cast films and used in this study. These samples were immersed in 6 mL of SS in 6 well plates and incubated at 37^{o}C with shaking at 150 rpm. Half of the supernatants (3 mL) were collected and stored at predetermined intervals and were then replaced with fresh SS. Imiquimod released into the supernatants was quantified by reverse phase high performance liquid chromatography (HPLC) using a Hitachi Primaide system equipped with a Kinetex C_{18} column. Imiquimod was separated from other constituents using a gradient mobile phase ranging from 20:80 to 80:20 acetonitrile to water containing 0.1%TFA at a flow rate of 1 mL/min, and detected at a wavelength of 242 nm. In addition to above studies, modified release studies were also conducted in subsequent experiments to address key problems. Changes such as full replenishment of release supernatants and/or replacing SS with acetate buffer (pH 4.0, 100 mM) were implemented.

Destructive erosion (mass loss) studies were performed in a similar way in 6 mL of SS. The initial sample weight (W_1) was recorded before the study, and final weight (W_2) was measured after drying the eroded samples at 43^{o}C overnight. Mass loss was calculated and plotted against erosion time.

A.3 Eudragit

A.3.1 Rationale

Previous studies (98) revealed that sustained release of imiquimod from 80:20 PVP:CMC formulation was caused by swelling of polymers and resultant slow erosion of films. However, rate of swelling was fast, reaching 245 % and 410 % by 10 and 60 minutes, respectively. Hence it was hypothesized that decrease in the swelling rate of these films may result in decreased erosion rate, thus increasing residence time. A hydrophobic polymer, Eudragit RL-PO (EUD; copolymer of ethyl acrylate, methyl methacrylate with low content of methacrylic ester with quaternary amine groups) was hence added to existing composition in an attempt to decrease swelling rate. Because majority of swelling in original films was contributed from CMC, no change in original composition was performed.

A.3.2 Film types

Four types of films with increasing EUD amounts were prepared and characterized. EUD was dissolved in methanol and added to PVP and CMC polymer solution as described above. EUD was added to reach 0,20,40,60 % w/w of total weight of PVP and CMC polymers.

A.3.3 Results and Discussion

A.3.3.1 Swelling:

Increased EUD concentration in the original PVP:CMC films resulted in lower swelling indexeseven though the amount of PVP&CMC remained constant in all types of films (Figure A.1) . Significant erosion of films was started from 200 minutes, and films in absence of EUD eroded quickly followed by completely erosion by 360 minutes. Incorporation of EUD resulted in almost stagnant swelling index of 200-250 from 300 minutes and were not completely eroded by the end of 7 hours. These results suggest the potential of EUD incorporated films to increase the erosion time of the films. However, it has to be observed that swelling studies were performed in static conditions, unlike release studies where dynamic conditions by shaking at 150 rpm were employed.

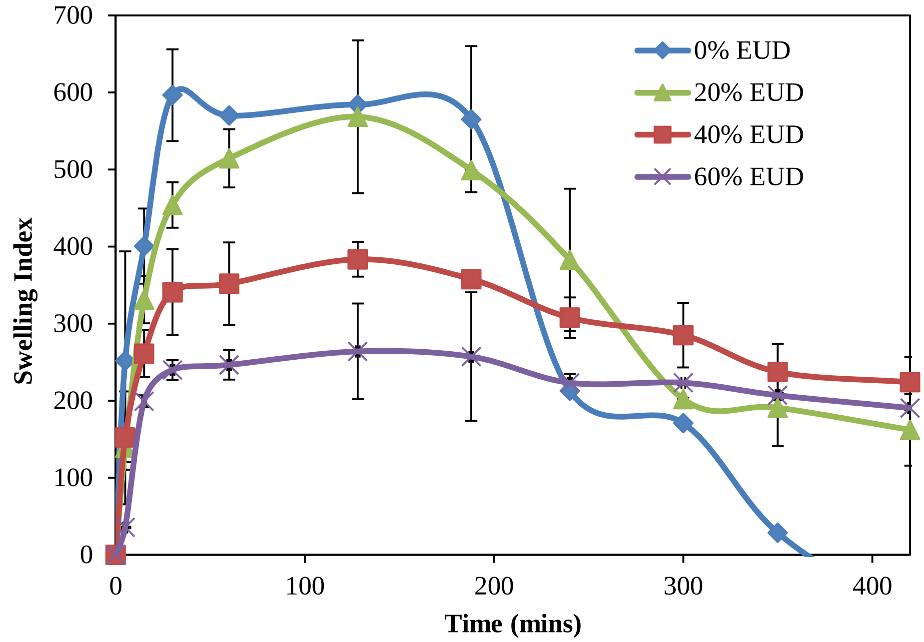


Figure A.1. Swelling profiles of mucoadhesive films with increasing amounts of EUD. Data are mean ± standard deviation (n≥3).

A.3.3.2 Ex vivo mucoadhesion time

Mucoadhesion time of all film types at two different thickness were tested on porcine buccal mucosa. Thicker version of films are 0.1 mm thicker than their thinner/normal versions. Incorporation of EUD did not significantly affect the *ex vivo* mucoadhesion time of normal films except 60% EUD type of film whose residence time was significantly decreased from 0 and 20% EUD (Figure A.2). *Ex vivo* mucoadhesion time followed biphasic trend with increase in EUD concentration in case of thicker films. *Ex vivo* mucoadhesion time increased at 20% EUD and started decreasing with further increase in EUD concentration. Statistical differences between mucoadhesion times of film types are shown in Figure A.2.

A.3.3.3 Release profiles

No difference in drug (imiquimod) release profiles was observed with incorporation of EUD polymer except that total amount of imiquimod released was observed to be higher with increase in EUD concentration (Figure A.3a). Drug was observed to be released for up to 8 hours in all types of formulations. However, 0%, and 20% EUD films were completely eroded by 210-240 minutes and 240-270 minutes, respectively. In contrary, 40% EUD films were broken in to 2-3 pieces, and 60% EUD films stayed intact at the end of study. In addition, 40% and 60 % EUD film types have also lost their adherence and separated from its backing layer by 90-120 minutes.

Release of the drug into release supernatants even after complete erosion of films in 0 % and 20% EUD film types may be attributed to collection and replenishment of half supernatants at each interval. Even though films were completely eroded, they may have still remained as invisible chunks of polymer in unremoved supernatants, and releasing drug slowly. Hydrophobic nature of the imiquimod may also have resulted in adsorption on plate surfaces to avoid SS, and getting solubilized after addition of fresh supernatants. Hence release studies were performed by fully replenishing the supernatants to confirm the hypothesis of delayed release of imiquimod from eroded chunks of polymer.

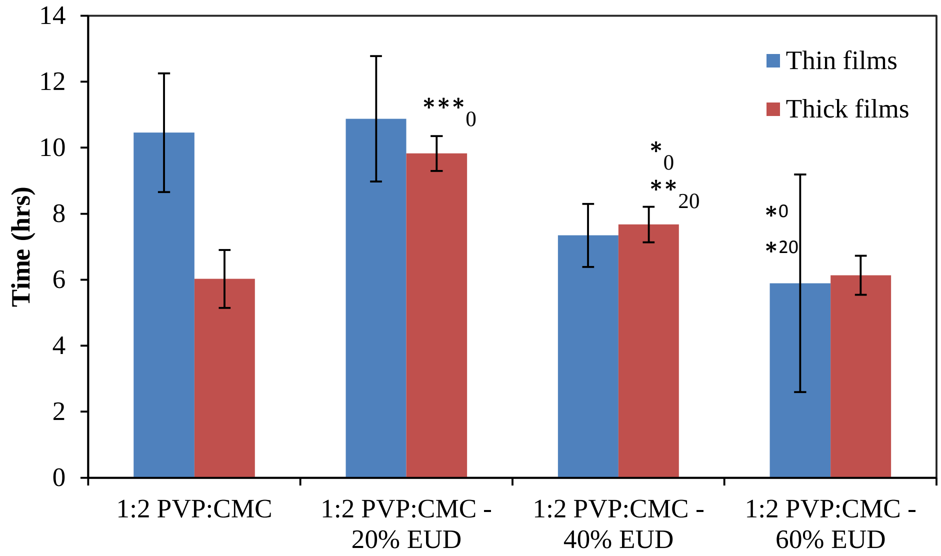


Figure A.2. *Ex vivo* mucoadhesion time of EUD incorporated films at two different thicknesses. Data are mean ± standard deviation (n≥3). *=p<0.05, **=p<0.01, ***=p<0.001

As hypothesized, amount of imiquimod extracted in to release supernatants decreased when release supernatants were fully replenished and discarded. Surprisingly, amount of imiquimod released between non EUD and EUD films was also significantly different in better sink conditions, and increased with increasing EUD concentrations (Figure A.3b). This increase may be due to increase in solubility of imiquimod owing to hydrophobic interactions between EUD and imiquimod. Because 60% EUD films stayed intact till the end of study, imiquimod was continuously being released.

Although release supernatants were fully replenished, very small amounts of imiquimod being released after complete erosion of the films, may be because of extremely hydrophobic nature of imiquimod, and thus its tendency to avoid SS by attaching to walls of tube and/or backing layer. These assumptions were confirmed when backing layers were dried after experiments and re-suspended in SS after 24 hours (results not shown).

A.3.3.4 Erosion studies

Mass loss of 20% and 60% EUD films with time were studied to understand the behavior of polymers and their erosion characteristics. Mass loss profile of 20% EUD films showed its complete erosion by 240-270 minutes, supporting visual observations (Figure A.4). However 60% EUD films lost a maximum of 62% of their mass only and remained intact by the end of study. Because PVP+CMC constitutes 62.5% mass (represented by horizontal lines in Figure A.4) in the later films, it shows diffusion of PVP and CMC with time leaving behind intact films made of EUD.

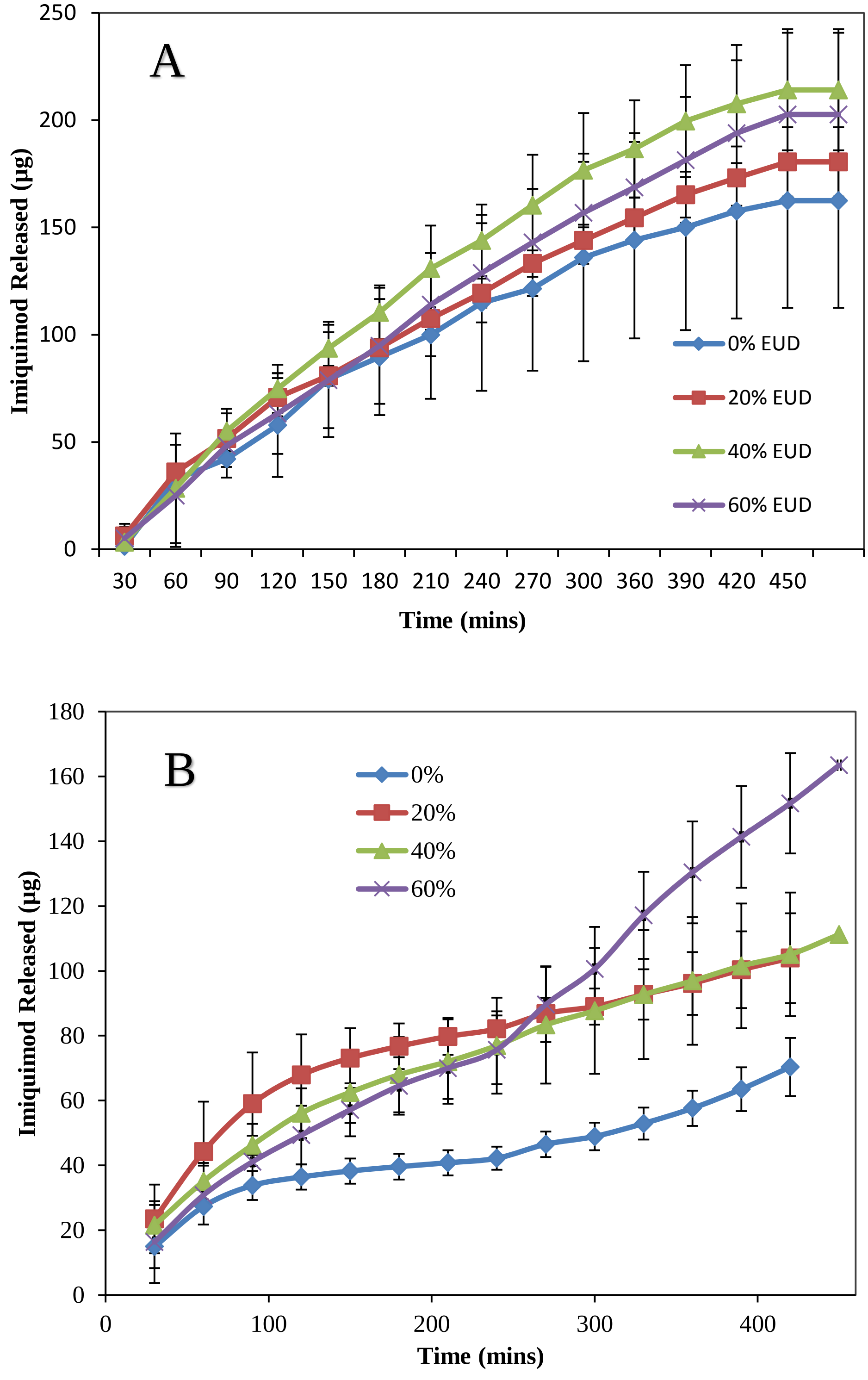


Figure A.3. Cumulative release profiles of all film types (A) in half replenished supernatants and (B) fully replenished supernatants.

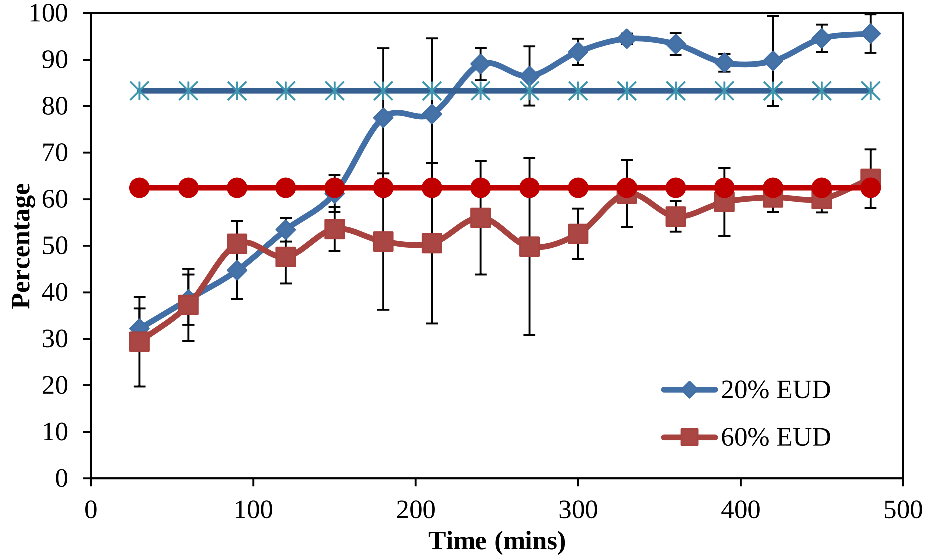


Figure A.4. Mass loss profiles of 20% and 60% EUD films. The horizontal lines represents the initial mass percentage of PVP+CMC of total polymer loaded in to films during fabrication

A.3.4 Conclusions

Incorporation of the hydrophobic polymer EUD increased erosion time with increase in its concentrations. However, diffusion of the hydrophilic polymers through intact matrix of EUD resulted in no difference between release profiles of different types of films. Although imiquimod was being released from 60% EUD films beyond 300 minutes in better sink conditions, loss of their adhesiveness to backing layer, and decrease in mucoadhesiveness to tissue hinders them from further development.

EUD increased release of drug into SS, and was more evident in only better sink conditions. Mucoadhesive films in *in vivo* conditions will have tight adherence to mucus tissue unlike free exposed surface *in vitro*. Such set up may have different sink conditions and its similarity to both tested conditions in this chapter is unknown. Permeability studies on Franz cell as performed in chapter 2 will further help determine the role of EUD in better release of drug.

A.4 Agar

A.4.1 Rationale and types of films

Saha et.al showed that addition of agar to PVP and CMC resulted in increase of erosion times up to 8 weeks and swelling indexes of 1400 (128-130). Hence agar was added to the current films, and quick erosion studies and *ex vivo* mucoadhesion time were calculated. Three types of films with increasing agar concentrations were prepared. Agar was added to reach 10, 25, and 100% of PVP + CMC weight and heated at 120 ^{o}C, 15 psi for 15-20 minutes. The resultant solution was then casted in to Teflon dishes and dried for different times.

A.4.2 Results and discussion

Film samples of 100% agar, stayed intact after 12 and 24 hours in 10 ml of SS. Cloudiness of release supernatants increased with time. However, samples lost adherence to their backing layer as early as 45 minutes. Hence *ex vivo* mucoadhesion time of films with different agar loading were determined before further studies. Compared to mucoadhesion time of 10 hours for normal 80:20 PVP:CMC films, addition of agar stayed for only 2, 3, and 0.75 hours for 10,25, and 100% agar loaded films. Backing layers of agar loaded films were also separated as early as 45 minutes.

A.4.3 Conclusion

Because adhesion of the films to mucus tissue is essential for release of drug, agar loaded films were not further studied even though they exhibited long erosion times

A.5 Increase in weight percentage and molecular weight of CMC

A.5.1 Rationale

Swelling studies and release profiles of 1:2 PVP:CMC and 2:1 PVP:CMC in chapter 2 showed that presence of more CMC increased swelling, erosion time and caused sustained release of drug up to 3 hours. Hence amount of CMC was further increased in this following study and resultant properties were investigated. In addition, CMC was replaced with its new variant of higher molecular weight and their swelling and release profiles were studied. The molecular weight of old CMC and new CMC were 250,000 and 700,000 Da. It was hypothesized that these changes in CMC increases, swelling, erosion time and release time of drug.

A.5.2 Film types

Three types of films were prepared as shown in Table A.1 with varied amounts of PVP and CMC. Amounts of other constituents such as polyethylene glycol remained same. Resultant solutions were mixed and casted in to Teflon dishes as described above.

A.5.3 Results and Discussion

A.5.3.1 Swelling studies

Swelling indexes of the films increased with increase in both mass, and molecular weight of CMC as hypothesized (Figure A.5). Films made up of high molecular weight CMC have swollen excessively and were difficult to handle after 340 minutes. While normal films completely eroded by 270 minutes, new film types remained intact up to 520 minutes. In addition to retaining their mass and uptake of water, significant swelling in radial direction was also observed with diameters of 2-3 times more than backing layer. Because new film types did not erode completely after 520 minutes, release profiles of these film types were further investigated to determine their capability to extend the drug release

Table A.1. Amounts of PVP and CMC in different types of films

Type	**PVP (g)**	**CMC (g)**
80:20	0.8	0.2
100:50	1.0	0.5
80:20 new CMC	0.8	0.2

A.5.3.2 Release studies

Release of imiquimod from the films was examined in acetate buffer (pH 4.0, 100mM), unlike SS, and release supernatants were completely replenished. Although SS is more similar to *in vivo* conditions, acetate buffer was used in this study only, to examine the potential of the new film types to extend drug release. Acetate buffer, possessing solubility of 2 mg/mL for imiquimod, may avoid any problems associated with hydrophobic nature of imiquimod, as observed during EUD studies. Release of imiquimod after complete erosion of films was observed during studies using SS (results not shown), and hence was difficult to interpret the effect of new film types.

Irrespective of the film type, imiquimod was released only up to 300 minutes (Figure A.6). No significant differences in release profiles of imiquimod was observed between film types. Although new film types stayed intact after 270-300 minutes, unlike 80:20 PVP:CMC films, imiquimod may have already diffused in to release supernatants.

A.5.4 Conclusion

Increase in mass and molecular weight of CMC increased erosion time and swelling of the mucoadhesive films. However, release of drug through their swollen matrix resulted in no improvement of extended drug release.

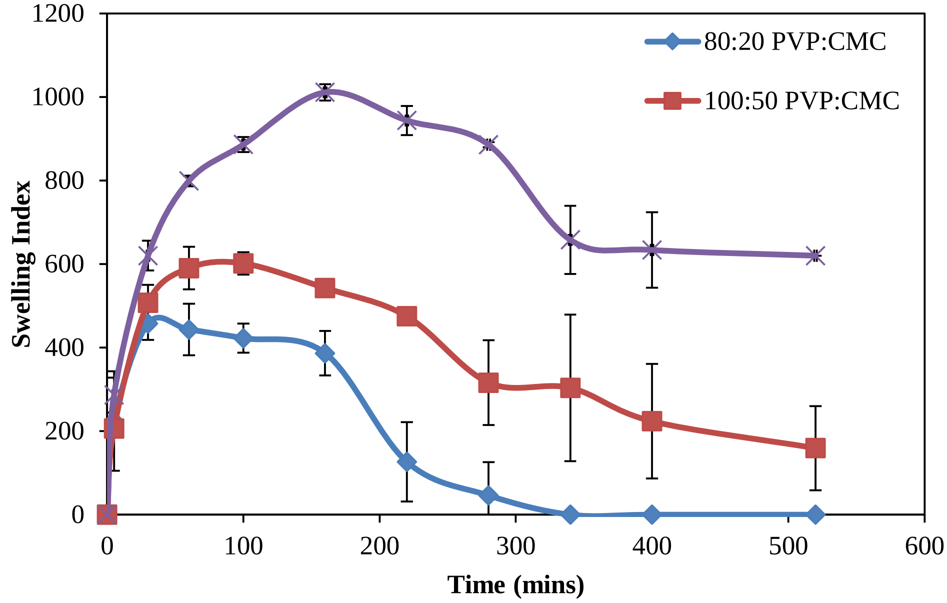


Figure A.5. Swelling profiles of mucoadhesive films with changes in CMC. Data are mean ± standard deviation (n≥3).

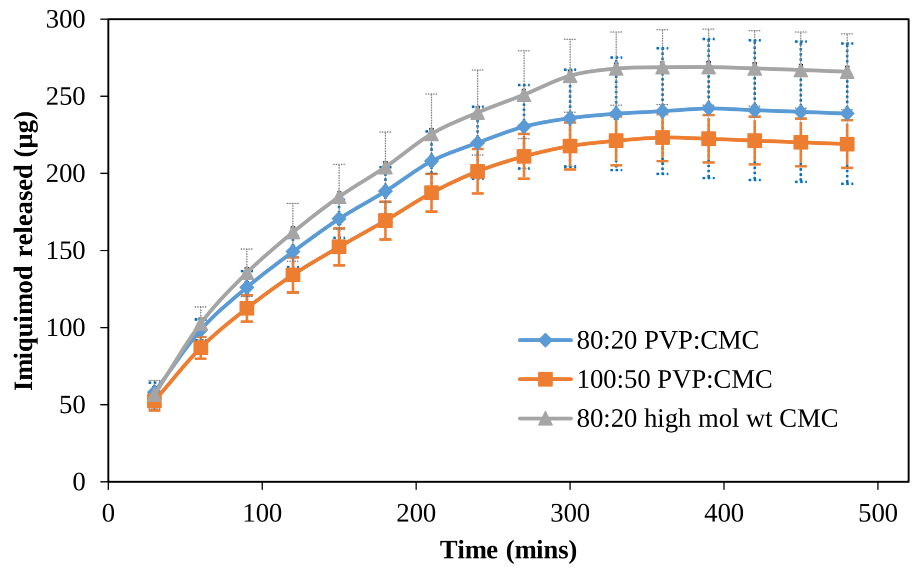


Figure A.6. Release profiles of mucoadhesive films with changes in mass and molecular weight of CMC. Data are mean ± standard deviation (n≥3).

A.6 Future Directions

Changes in physical design of system are desired to achieve balance in improvement of all essential properties, in order to continuously use current composition of polymers with minimal changes. Some of these changes may include incorporation of drug free perforated hydrophobic polymer layers to decrease swelling and erosion rates while maintaining adhesion to mucus tissue. Significant change in composition of polymers by addition of extra mucoadhesive polymer, such as polycarbophil or chitosan, can also enhance the residence time and drug release (131). Modification of polymers by conjugating molecules such as lectin, fimbriae can provide specific adhesion to cells (cytoadhesion), and thus increasing residence time (10). Such cytoadhesion of polymers helps in overcoming limitations of long residence time, caused by continuous mucus and saliva turnover (10).

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vaginal and rectal application. Daru : journal of Faculty of Pharmacy, Tehran University of Medical Sciences. 2012;20(1):67.

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PUBLICATIONS

- S.K. Ramineni, L.L. Cunningham Jr, T.D. Dziubla, D.A. Puleo, *Development of imiquimod loaded mucoadhesive films for oral dysplasia*, Journal of Pharmaceutical Sciences, 2013, 102 (2), 593-603.
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- S.K. Ramineni, L.L. Cunningham Jr, T.D. Dziubla, D.A. Puleo, *Competing properties of mucoadhesive films designed for localized delivery of imiquimod,* Biomaterials Science, 2013, 1(7), 753.

- J.C. Tharappel, S.K. Ramineni, D. Reynolds, D.A. Puleo, J.S. Roth, *Doxycyline impacts hernia repair outcomes,* Journal of Surgical Research, 2013, 184 (1), 699.

- R. Jain, S.K. Ramineni, N. Parekh**,** *IGIPT-Integrated Genomic Island Prediction Tool*, Bioinformation, 7 (6), 307, 2011
  Citations: PMID: 23402702, 23335123

- J.C. Tharappel, C.E. Bower, J.W. Harris, S.K. Ramineni, D.A. Puleo, J.S. Roth Roth J.S., *Doxycyline administration improves fascial interface in hernia repair*, Pending revision

- S.K. Ramineni, L.L. Cunningham Jr, C.W. Fowler, D.A. Puleo, *Development of mucoadhesive films for treatment of oral mucosal wounds and their testing on Oral Tissue Grafts*, submitted, 2014

- S.K. Ramineni, L.L. Cunningham Jr, T.D. Dziubla, D.A. Puleo, *Local Delivery of Imiquimod using Mucoadhesive Films*, submitted, 2014

- J.C. Tharappel, C.E. Bower, J.W. Harris, S.K. Ramineni, D.A. Puleo, J.S. Roth Roth J.S., *Doxycyline administration improves fascial interface in hernia repair,* Journal of Surgical Research, *2014, 186 (2), 688.*

- R. Jain, S.K. Ramineni, N. Parekh**,** *Integrated Genomic Island Prediction Tool (IGIPT)*, International conference on Information Technology, *ICIT'08*, 2008, 131-132.
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PRESENTATIONS

Oral

- "*Photodynamic Therapy",* National Conference, Chemical Engineers Meeting, Ichescon'06, Warangal, India, 2006

- "*Comparing Three Formulations of Imiquimod-Loaded Mucoadhesives for Treating Oral Dysplasia*", American Association for Dental Research 87 th General session and Exhibition, April, 2009

- *"Design of Mucoadhesive Patches for Treatment of Oral Dysplasia"*, Biomaterials Day, Cleveland, 2011

- *"Mucoadhesive films for regeneration of oral mucosa wounds",* Society for Biomaterials Annual meeting, Colorado, 2014

Poster

- *"Mucoadhesive Patches Delivering Imiquimod for Treatment of Oral Dysplasia"*, Society for Biomaterials Annual meeting, San Antonio, 2009

- *"Competing Properties of Mucoadhesive Films that Release Immune Response Modifiers"*, Society for Biomaterials Annual meeting, Seattle, 2010

- *"Mechanical Properties and Loading Techniques of Mucoadhesive Films for Treatment of Oral Dysplasia*", Society for Biomaterials Annual meeting, Orlando, 2011

- *"Localized Delivery of Imiquimod to Treat Oral Precancerous Lesions*", American Association for Dental Research General Session and Exhibition, Tampa, April, 2012

- *"Development of Mucoadhesive Films with Increased Residence Time for Treatment of Local Disorders*", Society for Biomaterials Annual meeting, Boston, 2013