

Moxifloxacin Loaded Ophthalmic Insert for Prophylaxis of Endophthalmitis

by

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Endophthalmitis is an inflammatory disorder that usually occurs in intraocular fluids or tissues involving vitreous and/or aqueous humors. It can be caused by bacterial, fungal, or other infecting microorganisms spreading from either inside or outside the body. As a serious eye disease, it is very likely to cause devastating consequences including irreversible vision loss even with prompt diagnosis and treatment. Endophthalmitis is a rare disease. The overall incidence rates of endophthalmitis are reported to be between 0.04% to 0.4%, but it remains a challenge to prevent the development of this disease. Moxifloxacin (MOX) is a broad spectrum, fourth-generation fluoroquinolone, and has been commonly used for prophylaxis of endophthalmitis. MOX is available as eyedrops, tablet and ocular injection, but they all have limitations such as low patient compliance, poor bioavailability and invasive administration route with potential risks. To address these problems, we proposed a moxifloxacin loaded, sustained-release ophthalmic insert that could deliver the drug locally and non-invasively for up to 24 h *in vivo*. In this study, we identified Eudragit® FS30D and Eudragit® EPO in ratio of 80%-20% as the optimal formulation of insert. The 10 mm inserts demonstrated a drug loading efficiency above 80%. Insert prepared with optimized formulation achieved a sustained release of MOX for 8 hours *in vitro*, and demonstrated good stability upon storage at 45°C, 75% relative humidity (RH), 25°C, 60% RH and 4°C. Meanwhile, a UPLC method for analysis of MOX was developed and validated in this study as well, which could be further optimized for analysis of MOX degradation in inserts. *In vitro* study showed that MOX loaded inserts were active against *Staphylococcus aureus* and *Escherichia coli*

and placebo inserts were non-toxic to human corneal limbal epithelial (HCLE) cells. Using a rabbit endophthalmitis model, the *in vivo* study showed that the prophylactic efficacy of MOX loaded insert was comparable with Vigamox[®] (0.5% moxifloxacin) eyedrop on a rabbit endophthalmitis model. In conclusion, we developed a moxifloxacin loaded ophthalmic insert that achieved a sustained release of drug for 8 hours and was non-toxic to HCLE cell. The insert also demonstrated satisfactory stability, promising anti-bacterial activity *in vitro* and prophylactic efficacy against endophthalmitis *in vivo*. This study was a good “proof of concept” that ophthalmic insert could be a potential platform for ocular sustained release.

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1.0 Introduction

1.1 Endophthalmitis and Its Incidence

Endophthalmitis is an inflammatory disorder affecting intraocular fluids or tissues involving vitreous and/or aqueous humors, which is caused by spread of bacterial, fungal, or other infecting microorganisms from either inside or outside the body.[1, 2] Although eye surgery is the most common source of introducing infectious pathogens, penetrating trauma in the eye or other adjacent tissues and spread of microorganisms from a non-ocular infection site can cause ocular infection and endophthalmitis as well. [3, 4] As a serious eye disease that is likely to cause devastating consequences including irreversible vision loss even with prompt diagnosis and treatment, endophthalmitis is always considered as an urgent medical emergency and needs to be treated as soon as possible.[5]

The clinical presentation of endophthalmitis may vary from person to person. In most cases, progressive vitritis and massive infiltration of inflammatory cells are found to be the hallmark of endophthalmitis. Other common symptoms include blurred vision (94%), hypopyon (85%), red eye (82.1%), hazy media (79%) and pain (74%), whereas swollen lid (34.5%) and light perception vision (26%) are seen less often among the patients.[6]

Endophthalmitis is a rare disease. The overall incidence rates are reported to be between 0.04% to 0.4%, however, this number may vary when it comes to a specific type of endophthalmitis, or the presence of other risk factors or medical conditions.[7, 8] Post-operative endophthalmitis accounts for the vast majority of all cases, and its incidence changes with time, which was 0.265% in the 2000-2003 period, 0.087% in the 1990s, 0.158% in the 1980s, and

0.327% during the 1970s.[9] Even though the incidence rate of endophthalmitis had been increasing from 20th century to early 21st century probably as a result of ocular surgery becoming much common than ever before, the occurrence has demonstrated a trend of decreasing in recent years, mostly owing to improved prophylaxis methods like use of intracameral antibiotics.[10]

1.2 Risk Factors and Pathogenesis of Endophthalmitis

In general, endophthalmitis is broadly divided into two categories: endogenous and exogenous.[1, 2, 5, 11, 12] Chronic metabolic diseases, immunosuppression, malignancy, intravenous drug abuse and intracorporeal foreign bodies are all risk factors that are considered to be associated with endogenous endophthalmitis. Cataract surgery, intravitreal injection and penetrating eye trauma are more dangerous than others such as filtering bleb in glaucoma and corneal infection as risk factors for causing exogenous endophthalmitis. Advanced age of 80 years or more is also a well-known risk factor for endophthalmitis. [5, 10, 11] According to the causative risk factors, endophthalmitis is categorized into several classifications including acute post-operative, chronic post-operative, post-injection, post-traumatic, bleb-related, endogenous and so on. Since the majority of post-operative endophthalmitis occurs after cataract surgery, it is also known as post-cataract endophthalmitis.[11, 12]

As an infectious disease, pathogens involved can be quite different, depending on the type of endophthalmitis. For example, Coagulase-negative *Staphylococci* are responsible for most cases of post-operative, post-injection and post traumatic endophthalmitis, and fungi infection causes half of the cases in keratitis-related endophthalmitis. Microorganisms such as *Staphylococcus*

aureus, *Streptococci*, *Pseudomonas* and *Escherichia coli* may also be found as causative pathogens for other types of endophthalmitis.[1, 3, 4, 11-13]

1.3 Endophthalmitis Prophylaxis Using Moxifloxacin

To avoid the grave outcome of endophthalmitis, physicians have been utilizing a series of techniques to prevent this disease, especially among patients who have cataract or other intraocular surgeries. Currently, many measures have been frequently adopted around the world, such as subconjunctival, intravitreal or intracameral injection of antibiotics post-operatively, irrigation of surgical site with filtered antibiotic solution and/or use of systemic antibiotics administered orally. Other than above-mentioned measures, pre- and post-operative use of topical antibiotics is another widespread prophylactic method usually used empirically by physicians in the US.[14-18]

Moxifloxacin (MOX), a broad spectrum, fourth-generation fluoroquinolone, has been commonly used for prophylaxis of endophthalmitis.[19, 20] Its mechanism of action involves disruption of bacterial DNA replication via inhibition of topoisomerase II (DNA gyrase) and topoisomerase IV, which are necessary enzymes for replication, transcription, and repair of bacterial DNA.[20] It is active against both gram-positive and -negative bacteria, and was approved for treatment of intraocular infection by the FDA since 1991. As one of the most comprehensively studied antibiotics, MOX has several advantages. In contrast to the other antibiotics used in prophylaxis of endophthalmitis, such as cefuroxime and vancomycin, MOX not only has lower toxicity, but also broader spectrum of antibacterial activity, greater potency against gram-positive pathogens, reduced susceptibility to resistance development, and most importantly, despite the mechanism being unclear, MOX does show better penetration into inflamed intraocular

tissues compared with first-, second- and third-generation of fluoroquinolone antibiotics, which is critical for topical administered antibiotics.[21-27] Moxifloxacin is commercially available as eyedrops (Vigamox[®]), oral tablets (Avelox[®]) and injections (Moxifloxacin PF[®]). In prophylaxis of endophthalmitis, eyedrops and injections are more commonly used because they can reach therapeutic concentration in intraocular tissues in shorter time, while oral tablets are rarely used alone and usually administered as a supplement of eyedrops or injections since limited amount of drug can reach and penetrate into the eye via systemic blood circulation.[15, 28] However, these dosage forms all have their own limitations. Eyedrops are well-accepted by patients and are easy to use, but they are faced with high dilution and efflux via tears. In addition, the eyedrop needs to be frequently administered from 4 times daily up to 1 drop/hour, and the prophylactic process may take 1 to 4 weeks, resulting in low patient compliance. [29] Tablets are popular with patients and physicians as well, but a very limited amount of drug can reach ocular tissues because of blood-retinal barrier, thus leading to inadequate concentration in the eye and low local bioavailability.[17, 30] Intraocular injections need to be administered once usually post-operatively, however, injections can potentially increase the risk of infection and blindness caused by injection site reaction, making them unfavorable for patients.[15, 31]

Currently, there is no commercially available sustained release product that can deliver moxifloxacin via topical, non-invasive route for prevention of endophthalmitis, and it is necessary to develop a novel local drug delivery system to overcome ~~forementioned~~ aforementioned limitations of conventional dosage forms such as poor bioavailability, low patient compliance and safety issues.

1.4 Treatment of Endophthalmitis

Endophthalmitis is rare, but its outcome may be devastating even with prompt diagnosis and treatment. As a result, clinicians tend to prevent the occurrence of endophthalmitis in the first place using previously mentioned prophylactic methods, rather than adopt treatment after the diagnosis of endophthalmitis. Even so, it has to be admitted that endophthalmitis probably could not be prevented completely and timely treatment is still required to improve visual outcomes of the disease.

In general, treatment of endophthalmitis includes topical eyedrops and intraocular injections of anti-microbials, systemic anti-microbials and surgical intervention such as pars plana vitrectomy (PPV), but clinicians may select one or a combination of treatment based on the type of endophthalmitis diagnosed or causative microorganisms.[3, 6, 7, 28, 32] For exogenous endophthalmitis, intravitreal antibiotics such as vancomycin (1.0 mg/0.1 ml), ceftazidime (2.25 mg/0.1 ml) and amikacin (0.4mg/0.1 ml) are usually the primary option, while topical antibiotics such as levofloxacin and moxifloxacin are considered as alternatives, supplemented by systemic antibiotics and PPV when necessary. Systemic and/or intravitreal corticosteroids may also be used to attenuate the inflammatory response to infection and avoid secondary damage. For endogenous endophthalmitis, systemic antibiotics are required, as the source of infection is located at non-ocular site within the body. Specifically, for endophthalmitis associated with fungal infection, surgical intervention combined with anti-fungal drugs administered systemically or intraocularly is necessary, and amphotericin B (5–10 µg/0.1 mL) as well as triazoles are used as primary therapeutic options.[3, 28]

1.5 Current Trends in Topical Delivery of Antibiotics for Ophthalmic Use

Novel approaches for topical delivery of antibiotics in ophthalmology is being explored nowadays, and researchers are mainly focusing on two directions.[33]

One involves the optimization of conventional dosage forms such as eyedrops and ocular injections, incorporating encapsulation of antibiotics in liposomes, micro/nanoparticles and nanocapsules, etc. For example, Das *et al.* developed polymeric nanoparticles fabricated by nanoprecipitation method using Eudragit® RL100 to deliver amphotericin B, and demonstrated those nanoparticles caused no irritation to the eye and had good stability upon storage at room temperature for 2 months; [34] Silva *et al.* formulated daptomycin loaded nanoparticles for topical delivery of the drug in treatment of bacterial endophthalmitis, and validated their antibacterial activity as well as stability in presence of lysozyme and mucins *in vitro*;[35] Koc *et al* reported intravitreal liposomal amphotericin B showed promising efficacy in treatment of *Candida* endophthalmitis. [36]

The other direction of exploring new approaches to deliver ophthalmic antibiotics topically that fascinates researchers is to load antibiotics in devices such as contact lenses and ocular inserts. There are already contact lenses commercially available for preparation of drug-loaded lenses using soaking technique, where contact lenses are immersed in drug solutions. [37] Hehl *et al.* demonstrated that gentamicin, ciprofloxacin and ofloxacin loaded in Acuvue® contact lenses had improved trans-corneal penetration and reached effective concentrations in the aqueous humor of patients.[38] Kakisu *et al.* investigated the uptake and the release of gatifloxacin and moxifloxacin from a newly synthesized hydrogel soft contact lens. They found the lens could continuously release antibiotics over 72 h, and improved the penetration of antibiotics into the eye. [39] Colo *et*

al. studied a erodible ofloxacin loaded poly(ethylene oxide) insert, and demonstrated its ability to improve bioavailability and potential in treatment of endophthalmitis.[40, 41]

1.6 Objectives and Innovations of Project

Endophthalmitis is a rare but dangerous disease. Although its incidence is low, once it develops, the visual outcome is usually poor if not treated in time, which makes prophylaxis of endophthalmitis essential in clinical practice. Moxifloxacin, as an antibiotic frequently used in prophylaxis of endophthalmitis, is available in dosage forms of eyedrop, tablet and ocular injection, but they all have limitations such as low patient compliance, poor bioavailability and invasive administration route with potential risks. Ocular insert has the advantage of delivering drug locally via non-invasive route, and it has potential to continuously release the drug for longer time, reducing administration frequency and improving patient compliance.

The objective of this project is to develop moxifloxacin loaded ophthalmic inserts that could continuously release the drug for up to 24 hours, which will limit the administration frequency to 1 insert per day, significantly improving patient compliance. The inserts should be able to easily retain in the eye of patient with no intolerable discomfort and removed by patient every day. In addition, we would have moxifloxacin loaded insert bearing equivalent efficacy to moxifloxacin eyedrops in prophylaxis of endophthalmitis. In this project, the inserts will be characterized for their weight, thickness, drug loading efficiency and drug release profile *in vitro*. Meanwhile, an ultra-high performance liquid chromatography (UPLC) method will be validated, which can be used for following stability tests of inserts. The anti-bacterial activity and

cytotoxicity of inserts will be tested *in vitro*, and their prophylactic efficacy against endophthalmitis will be evaluated *in vivo* using rabbit model.

This project first brought about a moxifloxacin loaded ophthalmic insert that could be administered topically and continuously release the drug for up to 8 hours. This novel formulation prepared with GRAS components demonstrated good stability, non-toxicity and anti-bacterial activity *in vitro*, and showed promising prophylactic efficacy of endophthalmitis *in vivo* using rabbit model.

2.0 Materials and Methods

2.1 Materials

Polymers for preparation of films (Eudragit[®] EPO, Eudragit[®] FS30D, Eudragit[®] RS30D and Eudragit[®] NM30D) were kindly provided as gift by Evonik[™], USA (Piscataway, NJ). Hydroxypropyl methylcellulose (HPMC) K4M and hydroxyethyl cellulose (HEC) Natrosol[™] 250 were kindly provided by Dr. Rohan at University of Pittsburgh, School of Pharmacy. Polyethylene glycol 400 (PEG 400), was purchased from TCI America (Portland, OR). Moxifloxacin hydrochloride (eMolecules[™]), hyaluronic acid (Acros Organics[™]) and other chemicals used in this study were all of analytical grade and purchased from Thermo Fisher Scientific (Waltham, MA) unless specified otherwise. All chemicals used for mobile phase of UPLC were LC/MS grade. Media used for cell culture including Keratinocyte serum free media (KSFM) and Dulbecco's modified Eagle media: Nutrient mixture F-12 (DMEM/F-12) were also purchased from Thermo Fisher Scientific (Waltham, MA). Newborn Calf Serum (NCS) was purchased from Sigma-Aldrich (St. Louis, MO). PBS and Cell culture grade DMSO were purchased from Corning[™] USA (Corning, NY).

2.2 Methods

2.2.1 Preparation of Inserts

To obtain inserts of desired size, films were first prepared using a solvent casting method. For MOX loaded films, Eudragit[®] FS30D and moxifloxacin were dissolved in distilled water containing 0.8 g of PEG 400 and mixed under magnetic stirring at 700 rpm for 2 h. Then HPMC K4M and HEC (Natrosol[™] 250) were added to the mixture and left under stirring for another 2 h to ensure complete dissolution. After that, the last Eudragit[®] polymer (EPO, NM30D or RS30D) was added to the solution and mixed under stirring overnight. The prepared solution was poured into micropatterned mold and degassed using vacuum pump to remove air bubbles. Finally, the mold containing poured solution was kept in oven overnight at 70 to 75°C for the films to form. Films were then peeled off from the mold on the next day, weighed and wrapped in aluminum foil for further experiments. Placebo films were prepared without moxifloxacin using the same procedure. The micropatterned mold was casted using templates with micropatterns in shape of circle, square and triangle, diameters of 100 or 200 μm , and intervals of 100 or 200 μm between neighboring micropatterns.

MOX loaded HA films were prepared using similar method with some modifications. Briefly, MOX was dissolved in water containing equal amount of HA and 0.8 g PEG 400, and mixed under magnetic stirring at 700 rpm for 2 h. Eudragit[®] FS30D was then added to the solution and mixed under stirring for another 2 h. HPMC K4M and HEC (Natrosol[™] 250) were added to the solution and left under stirring for 2 more hours. Finally, Eudragit[®] EPO was added to the solution, and the solution was kept under stirring overnight. The complete solution was poured into micropatterned mold, degassed on the following day and left in drying oven overnight at 70

to 75°C for the films to form. Films were peeled off from the mold on the next day, weighed and wrapped in aluminum foil for further experiments.

Inserts for following tests and analysis were cut from the film using punches of different sizes (5/8/10 mm) according to experimental design, and films were all freshly prepared within 24 hours before each experiment.

2.2.2 Analysis of Moxifloxacin

For drug loading and drug release tests, MOX was analyzed using UV method on NanoDrop One^C (Thermo Scientific™, Waltham, MA). A full spectrum scan (190-800 nm) was performed first in order to decide the optimal wavelength for UV detection of MOX, and 290 nm was determined to be the detection wavelength for following analysis. MOX standard calibration curve was set up from 1 µg/ml to 200 µg/ml. For this, MOX stock of 1 mg/ml was prepared by dissolving 50 mg of MOX in 50 ml of distilled water using volumetric flask. The stock was diluted to 1, 2.5, 5, 10, 15, 20, 25, 50, 100, 150 and 200 µg/ml. UV absorbance of each concentration was measured at 290 nm using NanoDrop One^C on UV-Vis mode.

2.2.3 Drug Loading and Loading Efficiency

All MOX loaded inserts were tested for drug loading and loading efficiency. The target drug loading used for drug release tests was 300 µg/10 mm insert (30 mg/film), which was based on the estimated dosage for 24 hours (12 drops per day of 5% w/v MOX eyedrop, 50 µl per drop).[42, 43] To match the dosage administered in rabbit endophthalmitis prophylaxis model (5% MOX eyedrop, 10 drops in total, 50 µl per drop), the inserts tested aimed to have drug loading of 2.5

mg/8 mm insert (600 mg/film) and 2.5 mg/5 mm (1200 mg/film). Inserts used for validation of antibacterial activity *in vitro* by Kirby-Bauer test aimed for drug loading of 5 µg and 8 µg per 5 mm insert, which was in the range of the concentration of MOX standard solutions used in the experiment. Inserts were cut from films using punches of different sizes according to the subsequent tests that inserts were subject to, weighed and transferred in falcon tubes containing 10 ml of distilled water. The tubes were then sonicated for 2 h at ambient temperature to ensure complete dissolution of loaded MOX. The obtained solutions were centrifuged at 1500 rpm for 5 min. Supernatant was diluted to appropriate concentration within the linear range of calibration curve, and its UV absorbance was measured at 290 nm using NanoDrop One^C on UV-Vis mode. Drug loading and loading efficiency were calculated using the following formulas:

$$\text{Drug loading} = \frac{\text{Drug loaded in disk}}{\text{Weight of disk}} \times \text{Weight of film}$$

Loading Efficiency %

$$= \frac{\text{Drug loading} \times \text{Total weight of ingredients}}{\text{Weight of drug} \times \text{Weight of solution poured in mold}} \times 100\%$$

2.2.4 MOX Release from Inserts *In Vitro*

Inserts were cut from films using 10 mm punch, weighed and put into 4-ml capped clear glass vials. PBS (pH 7.4) was selected as release medium, and 1 ml of PBS was added to the vial. Vials were then kept in a shaker at 100 rpm, 37°C. At specific time intervals (5 min, 15 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 6 h, 8 h and 24 h), 400 µl of release medium was transferred into a centrifuge tube, and an equal amount of fresh PBS was replenished in the vial. Samples were

diluted to appropriate concentrations within the linear range of calibration curve and their UV absorbance was measured at 290 nm using NanoDrop One^C on UV-Vis mode. Drug release profile was depicted by plotting cumulative release % of MOX *versus* time.

2.2.5 Characterization of MOX Inserts

Films were cut into inserts of 10 mm diameter using a punch, and inserts were weighed by analytical balance. Thickness of films was measured using a thickness gauge (Mitutoyo, Japan). The thickness of each film was measured at five different locations over the sampling area. Results are shown as mean values and SDs of each film. The micropattern of films were observed under microscope at different magnifications.

2.2.6 Development of Stability-Indicating UPLC Method for Analysis of Moxifloxacin

2.2.6.1 Instrument

An Ultra-high Performance Liquid Chromatographic (UPLC) system (WatersTM Acuity®) with Acuity® UPLC BEH C18 Column (130Å, pore size 1.7 µm, 2.1 mm X 100 mm), equipped with TUV detector set at 290 nm for the analysis and MassLynx software for data processing.

2.2.6.2 Preparation of mobile phase and standard working solution

The solvent system of method consisted of two components, mobile phase A (95% water, 5% acetonitrile, 0.1% formic acid, 2mM ammonium formate) and mobile phase B (5% water, 95% acetonitrile, 0.1% formic acid, 2mM ammonium formate). Mobile phase A was prepared by first mixing 950 ml of water with 50 ml of acetonitrile, then 1ml of formic acid was added to the

solution together with 0.126g of ammonium formate. Mobile phase B was prepared by first mixing 950 ml of acetonitrile and 50 ml of water, then 1ml of formic acid was added to the solution together with 0.126g of ammonium formate

Moxifloxacin stock solution of 1 mg/ml was prepared by dissolving 50mg moxifloxacin in deionized water using 50 ml volumetric flask. Moxifloxacin working solution ranging from 0.5 µg/ml to 200 µg/ml was prepared by dilution of the stock solution in deionized water.

2.2.6.3 Chromatographic condition

The chromatographic condition of system was similar to reported method, with some modifications.[44] The chromatographic separation was achieved using an Acquity® UPLC BEH C18 Column (130Å, pore size 1.7 µm, 2.1 mm X 100 mm) and a gradient elution method. The flow rate was set at 0.3 ml/min and column temperature was maintained at 40°C. Eluent was monitored at 290 nm for detection of moxifloxacin. Injection volume was 1 µl and runtime was set to 8 min. Prior to injection, the column was washed with methanol and equilibrated with a mixture of 95% mobile phase A and 5% mobile phase B. Programing details of the gradient elution method is listed in **Table 1**.

Table 1 Program of gredient elution method

Time (min)	Mobile Phase A	Mobile Phase B
0	95%	5%
1	95%	5%
1.5	60%	40%
3	60%	40%
4	0%	100%
5	0%	100
5.10	95%	5%
8	95%	5%

2.2.6.4 Calibration curve of moxifloxacin

Moxifloxacin standard working solutions from 0.5 µg/ml to 200 µg/ml were injected to the system, and chromatogram was recorded. The Calibration curve was made by plotting peak area on y-axis against respective concentration of working solution on x-axis.

2.2.6.5 Method validation

The method was validated in terms of different parameters including linearity, precision, accuracy and stability in accordance with literatures.[44-49]

Linearity of response was established by plotting concentrations of working solutions against peak area. Moxifloxacin standard working solution ranging from 0.5 µg/ml to 200 µg/ml were analyzed as per test method. A regression curve was made and the correlation coefficient was determined.

Accuracy of method was evaluated at selected concentrations, and recovery % was calculated from the calibration curve.

Precision of method was determined by intra-day and inter-day precision. Intra-day precision was determined by performing 5 injections of each quality control samples of selected concentrations at 5 timepoints on the same day. Inter-day precision was determined by performing injections of the quality control samples of selected concentrations on 4 consecutive days.

Solution stability was determined by performing injections of quality control samples stored at room temperature for 24 h, 4 °C for 72 h and freeze-thawed for 3 times.

2.2.7 Stability of MOX in inserts

The stability of MOX in inserts was conducted as per the ICH guidelines.[50] The MOX loaded inserts were stored under 3 conditions, 45°C, 75% relative humidity (RH), 25°C, 60% RH and 4°C in refrigerator. Study design details are as shown in **Table 2**. For each group, samples to be tested at one timepoint were packed and sealed in an aluminum pouch and stored in stability chamber until the day of test. Samples were weighed before being stored in the stability chamber, and were weighed again at testing timepoint. The inserts were tested for their drug release profile and variation of weight during the storage period.

Table 2 Group design of stability test

Group	Condition	Test timepoints
1	40°C, 75% RH	Day 0, wk 1, 2, 4, 6, 8, 10 and 12
2	25°C, 60% RH	Day 0, month 1, 2, 3, 6 and 12
3	4°C in refrigerator	Day 0, month 1, 3, 6 and 12

2.2.8 Validation of Anti-bacterial Activity of MOX Inserts *In Vitro*

The MOX loaded inserts were prepared using solvent casting method, which included a step of oven drying at 70-75°C for 24 h. There was risk that MOX might lose its anti-bacterial activity at high temperature, and whether or not MOX remained active in inserts had to be determined. MOX standard solutions and drug loaded film disks of 5 mm diameter containing 5 µg and 8 µg

of MOX were used to validate the anti-bacterial activity of inserts *in vitro* by Kirby-Bauer test.[51, 52] This experiment was conducted by Dr. Kowalski's lab, University of Pittsburgh, School of Medicine, Department of Ophthalmology. One-hundred-and-fifty-mm Mueller-Hinton agar plates were each seeded with lawns of *Staphylococcus aureus* (ATCC strain 25923) and *Escherichia coli* (ATCC strain 29922), and kept in the incubator for overnight growth one day before testing. On the day of testing, 25 μ l of MOX standard solution at each concentration was added to sterile 6-mm blank paper disks, and paper disks were immediately transferred onto agar plates seeded with *S. aureus* and *E. coli*. Blank paper disks were instilled with 25 μ l of 4, 20, 40, 80, 120, 160, 200 and 500 μ g/ml MOX standard solutions, which contained 0.1, 0.5, 1, 2, 3, 4, 5 and 12.5 μ g MOX, respectively. Five-mm inserts loaded with 5 μ g and 8 μ g of MOX was used for this test to match the drug loading range of paper disks. Five-mm drug loaded inserts were also placed onto agar plates seeded with bacteria simultaneously with paper disks. All agar plates were then incubated overnight at 37°C. Zone of inhibition was measured on the next day, and zone sizes of MOX standard solutions were used to produce concentration equations using Quadratic Fitted Line Plot Regression Analysis of antibiotic concentration versus zone size for the MOX standards for each bacterial strain (Minitab Version 16, State College, PA). Experiments were carried out in duplicate, and zone of inhibition was measured by two readers each time. The approximate amount of active MOX contained in the disks were then determined using above-mentioned equation.

2.2.9 Cytotoxicity Assay of Placebo Inserts

2.2.9.1 Cell culture

Human corneal limbal epithelial (HCLE) cells, an immortalized cell line, was obtained from Dr. Shanks' lab, and used for cytotoxicity study. Cells were cultured as previously

described.[53, 54] In brief, HCLE cells were grown in T-75D flasks in Keratinocyte- serum-free (KSFM) medium. KSFM medium was supplemented with 25 µg/mL bovine pituitary extract (BPE) and 0.2 ng/mL epidermal growth factor (EGF) that came together with medium from manufacturer. One-percent Pen/Strep was added to complete KSFM medium to prevent microorganism contamination. DMEM/F12 medium supplemented with 20% Newborn Calf Serum and 20% cell culture grade DMSO was used for cell preservation.

2.2.9.2 Determination of cytotoxicity

The cytotoxicity of placebo inserts was assessed using resazurin assay on human corneal limbal epithelial (HCLE) cells.

Ten-percent resazurin medium used for the assay was prepared with 0.1 mg/ml resazurin PBS stock solution and KSFM culture medium. In short, 5 mg of resazurin was dissolved in 50 ml of PBS to obtain 0.1 mg/ml stock solution. Then 5 ml resazurin stock was mixed with 45 ml of KSFM culture medium to make 50 ml of 10% resazurin medium. Both resazurin stock and medium were wrapped with aluminum foil and kept at 4°C in refrigerator until use.

Cells were seeded in 24-well tissue culture plate at a density of 0.2 million cells per well. One ml KSFM medium was added to each well and cells were allowed to adhere to the bottom of plate to form a monolayer in the incubator for 24 h at 37°C, 5% CO₂. Fresh medium was replaced in each well on the next day. Films were cut into inserts of 5 mm diameter, sterilized under UV for 30 min on each side, and placed in each well with their pattern side facing the bottom of plate. The cells were then incubated for another 24 h with inserts. After that, film inserts and medium were removed. Cells were washed with calcium- and magnesium-free PBS before addition of 10% resazurin medium (1 ml per well). The plate was then kept in incubator for 2 to 3 h until the color of medium had changed. Finally, 100 µl of medium from each well was transferred to a 96-well

plate, and its fluorescence absorbance was measured using a plate reader (Spectrum M5E) at excitation wavelength of 560 nm, emission wavelength of 590 nm. Fresh resazurin media was used as blank, and resazurin media from wells with untreated cells was used as control. Each measurement was performed in triplicate and data was processed using SoftMax® Pro software.

2.2.10 Determination of Insert Retention and Prophylactic Efficacy *In Vivo*

2.2.10.1 Animals

Animal studies of MOX inserts were performed by Dr. Kowalski's lab, University of Pittsburgh, School of Medicine, Department of Ophthalmology. Fifteen New Zealand White Rabbit weighing 1.1 to 1.4 kg from Charles River's Oakwood Canadian rabbitry were used for the study. Upon arrival, Rabbits were allowed to acclimatize to the facility for 7 days prior to experiment. All experiments conformed to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research, and were performed in accordance to IACUC Protocol #21018413 "Endophthalmitis prophylaxis with controlled-release of antibiotic from an ocular film insert" approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.2.10.2 Determination of insert retention in un-taped rabbit eye

The retention of inserts was tested first using 5 mm placebo inserts of different micropatterns (Unpatterned, Square 100 D100, Triangle 100 D100 and Square 200 D200) by two experiments. The micropattern designation was defined by its shape, size and distance between micropatterns. For example, Square 100 D100 indicates that the shape of micropattern is square, its size is 100 μm by 100 μm , and the distance between two square micropatterns is 100 μm .

This experiment was conducted to determine whether inserts will stay in rabbit eyes without taping eyes shut overnight after being placed into the cul-de-sacs of rabbit. Briefly, the left eyes were anesthetized with two drops of 0.5% proparacaine before 5 mm inserts of different patterns were placed into the cul-de-sacs of left eyes, with their patterned sides against the eyeball. The rabbits were then returned to their cages and their eyes were examined every 2 hours for 3 examinations on the day of insertion to determine the retention of inserts. The eyes were formally examined again for irritation using flashlight examination and grading using the Draize scale following the last insert examination. [55] Twenty-four hours later, the eyes were examined again to determine the retention of inserts and irritation. Rabbits were returned to their cages and allowed to recover for 24 h before commencing the next experiment. Group design of the experiment is shown in **Table 3**.

Table 3 Group design of insert retention (eyes un-taped)

Group	Inserts	N Eyes	Rabbit Numbers
I	Unpatterned	4	1-4
II	Squares 100D100	4	5-8
III	Triangles 100D100	4	9-12
IV	Squares 200D200	3	13-15

2.2.10.3 Determination of insert retention in taped rabbit eye

This experiment was conducted to determine whether 5 mm inserts will stay in rabbit eyes overnight after placing them into the cul-de-sacs with taping the eyes shut or whether pre-wetting the inserts before placement will allow better retention in open eyes. The experimental procedure was similar to the first experiment with some modifications. Fur around the left eyes of rabbits was shaved one day prior the experiment, and inserts were hydrated in sterile water for 10-15 seconds before placing them into the eyes with their patterned sides against the eye. All inserts were placed into the cul-de-sacs of left eyes using forceps and pressure was applied to the inserts

against the eyes. According to the group design, the tested eyes were either taped shut using Steri-strips or left open, and only rabbits with eyes taped were fitted with modified buster collars to prevent them from wiping eyes. The status of eyes was monitored throughout the experiment to ensure the Steri-strips were not removed from taped shut eyes and collars were not removed or twisted. If tapes were displaced, the eye would be checked whether the insert retained, and the eye would be re-taped if so. If collars were removed or twisted, they would be reattached, reoriented or replaced. All tapes and collars were removed prior to the final examination of irritation and insert retention after 24 h of insertion. Rabbits were returned to their cages and allowed for recovery until the next experiment. Group design of the experiment is shown in **Table 4**.

Table 4 Group design of insert retention (eyes taped)

Group	Inserts	Eyes Taped (T) Or Open (O)	N Eyes	Rabbit Numbers
I-T	Unpatterned	T	4	1-4
I-O	Unpatterned	O	3	5-7
II-T	Squares 100D100	T	4	8-11
II-O	Squares 100D100	O	4	12-15

2.2.10.4 Evaluation of Prophylactic Efficacy of MOX Inserts *In Vivo* in Endophthalmitis

Rabbit Model

In vivo efficacy study was conducted to determine the efficacy of inserts loaded with 2.5 mg moxifloxacin placed into the cul-de-sac compared with standard topical antibiotic prophylaxis with 0.5% moxifloxacin solution (Vigamox®) for the prevention of *Staphylococcus aureus* endophthalmitis in the New Zealand White rabbit antibiotic prophylaxis model.

Fur around the right eyes of rabbits was shaved to reveal bare skin prior to experiment, and rabbits were divided into 3 groups and treated as shown in **Table 5**. All rabbits were anesthetized with 40 mg/kg of ketamine & 4 mg/kg of xylazine administered intramuscularly 20 minutes prior

to bacterial inoculation. Rabbits were treated with one dose of ketoprofen (1.5 mg/kg) before bacterial inoculation, and 25 µl of inoculum containing 6.24×10^4 CFU of clinical endophthalmitis isolate of fluoroquinolone-susceptible *Staphylococcus aureus* (E253) was inoculated into the anterior chamber near the central cornea, followed by topical anesthesia with 2 drops of proparacaine. For the group treated with Vigamox® (**MOX**, Group I), one drop of Vigamox® was instilled into the eye at 60 min, 45 min, 30 min, 15 min and 0 min prior to bacterial inoculation, and 0 min, 2 h, 6 h, 19 h and 22 h post bacterial inoculation. After bacterial inoculation, MOX inserts and placebo inserts were placed into the cul-de-sac of rabbit eyes in moxifloxacin insert group (**MOX-I**, Group II) and placebo insert control group (**CON-I**, Group III). Eyes in Group II and Group III were taped shut using Steri-strips and fitted with modified buster collars to prevent them from wiping their eyes after inserts had been placed.

Table 5 Group design for evaluation of prophylactic efficacy

Group	Rabbit Numbers	Treatment	N rabbits	N eyes
I MOX	1-5	0.5% Moxifloxacin (Vigamox®) (1 Drop of 50 µl) 60, 45, 30, 15, & 0 minutes prior to inoculation; Immediately post-inoculation; 4 times over the next 24 hours	5	5
II MOX-FI	6-10	Moxifloxacin Film Insert (MOX-I) 60 minutes prior to inoculation	5	5
III CON-FI	11-15	Control (Placebo) Film Insert (CON-I) 60 minutes prior to inoculation	5	5

Twenty-four hours after the bacterial inoculation, eyes were photographed, examined and graded for clinical signs of endophthalmitis using the slit lamp. Rabbits were sacrificed with an overdose of intravenous Euthasol solution following intramuscular 40 mg/kg of ketamine & 4 mg/kg of xylazine for systemic anesthesia. Aqueous humor and vitreous humor taps were then

performed on the inoculated eyes to retrieve samples for bacterial culture. Samples were seeded onto blood agar plate or trypticase soy broth, and examined for bacterial growth 24-48 hours later. Positive microbiological endophthalmitis is considered if positive growth of *Staphylococcus aureus* is observed in sample either seeded on the blood agar plate or in trypticase soy broth.

Clinical grading of eyes was performed by a masked physician using slit lamp examination. Discharge, conjunctival/sclera injection, limbal injection, hypopyon, iritis, Anterior Chamber (AC) cell, AC flare, fibrin production and corneal infiltrate were graded each based on its severity on a scale of 0-3. The development of endophthalmitis was defined by either a total anterior chamber score (total of hypopyon, iritis, fibrin production, AC Cell, and AC Flare) of 3 or more, positive bacterial culture, or the clinical diagnose of clinician.

3.0 Results and Discussion

3.1 Analytical Method of Moxifloxacin

A full spectrum scan (190-850 nm) was performed using Nanodrop One^C to confirm the optimal wavelength for UV detection of MOX. The spectrum is depicted in **Figure 1**, indicating the peak was located between 285-295 nm. As such, 290 nm was determined as the wavelength for UV detection of MOX in following analysis of this study. MOX standard calibration curve was established afterwards, shown in **Figure 2**.

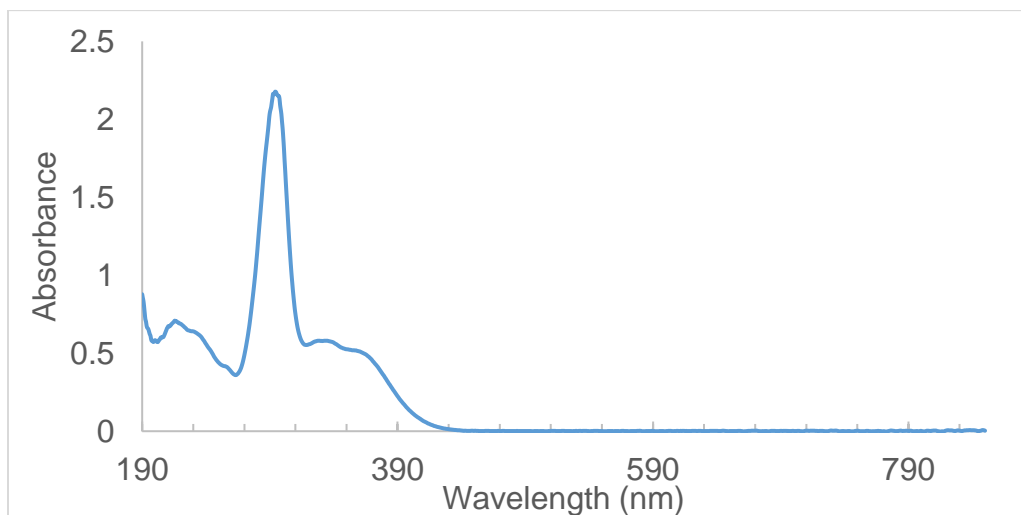


Figure 1 Full UV-Vis Spectrum of Moxifloxacin (190-850 nm)

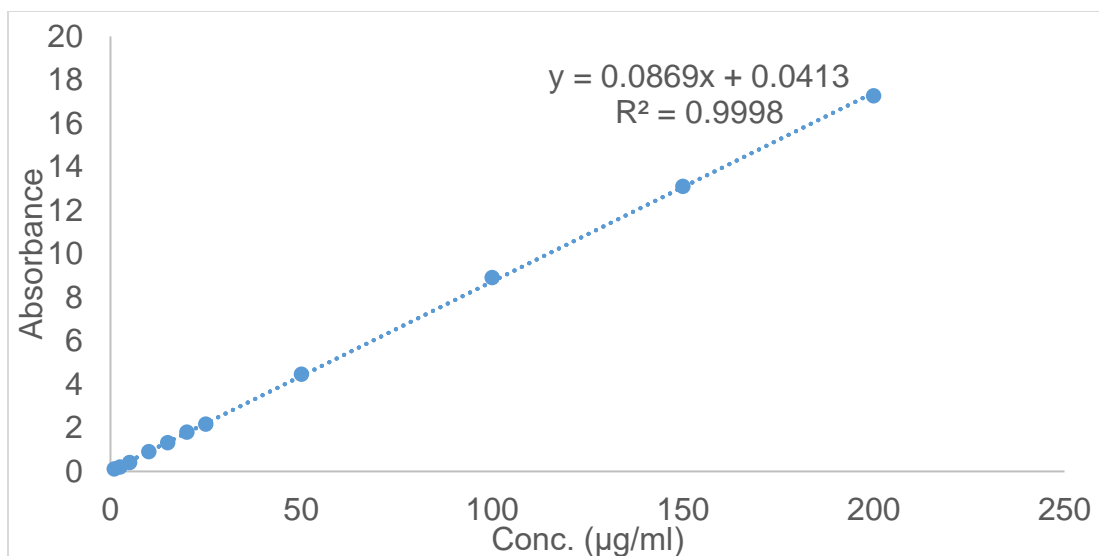


Figure 2 Calibration curve of moxifloxacin (1-200 µg/ml)

3.2 Formulation Optimization of Inserts

Moxifloxacin (**Figure 3**) is slightly soluble in water with an estimated solubility of 1.146g/L at 25°C, but its hydrochloride form has a much higher solubility of 19.6-32.4 mg/ml at 20-50°C. [56, 57] The main challenge in sustaining the release of moxifloxacin hydrochloride is its high solubility in water. Without release modifiers, it is expected that moxifloxacin will be completely released in a short time once the carrier containing the drug enters a aqueous environment such as tear fluids on ocular surface. To ensure that MOX is not completely and immediately released, the inserts should be able to maintain their integrity and not dissolve in tear fluid or on ocular surface at pH 7, and for this reason, the combination of polymers used in the formulation of inserts always included one polymer that does not dissolve at pH 7 (EPO, RS30D or NM30D).[58, 59] Moxifloxacin hydrochloride is stable to acidic hydrolysis, aqueous hydrolysis, thermal stress, and photolytic stress, but relatively unstable to basic hydrolysis and

oxidative stress.[47] As the polymer solution is of neutral pH, fabricating MOX loaded inserts using solvent casting method is expected not to affect the stability of MOX in inserts.

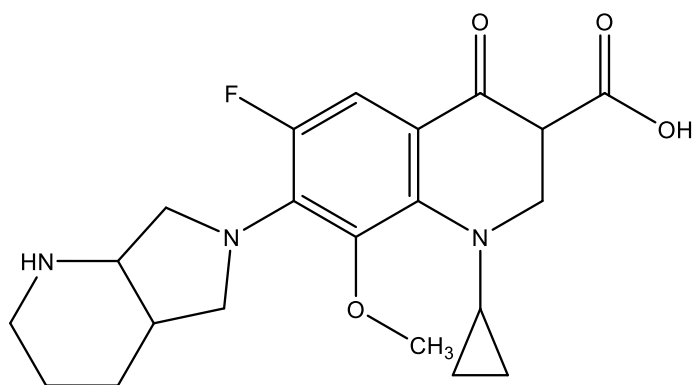


Figure 3 Structure of moxifloxacin

Ocular drug delivery is faced with problems caused by multiple physiochemical barriers such as dilution and efflux via tear, cornea, conjunctiva and sclera restricting drug permeation and penetration into ocular tissues, as well as blood-aqueous/retina barriers.[60, 61] Ophthalmic films have potential to overcome those limitations because of flexibility in their composition, and various additives can be incorporated to the formulation in order to modify the properties of film. In the current study, the formulation of film comprised of two major components, polymers that formed the matrix for sustained release, and plasticizers with viscosity enhancing properties.

All components in the formulation were graded as GRAS. Several Eudragit[®] polymers were tested in this study. Eudragit[®] is the brand name covering a diverse range of polymethacrylate-based copolymers. These polymers can be anionic, cationic, or neutral, and their properties differ depending on the methacrylic acid and methacrylic/acrylic esters or their derivatives. Some Eudragit[®] polymers display pH-dependent solubility to varying degree.[62] Eudragit[®] FS30D is an anionic, carboxylate containing poly(meth)acrylate copolymer soluble above pH 7, which has been used as enteric coating; Eudragit[®] EPO is a cationic, amine containing poly(meth)acrylate polymer soluble below pH 5, which has been used as film coating; RS30D is

a polymer composed of poly(ethyl acrylate, methyl methacrylate, trimethyl aminoethyl methacrylate chloride) and NM30D is a polymer composed of Poly(ethyl acrylate, methyl methacrylate) plus PEG stearyl ether. Both RS30D and NM30D are non-ionic and insoluble in aqueous solution, and have been used for sustained release formulations.[62-66] HPMC K4M is a member of hydroxypropyl methylcellulose family, which is one type of cellulose ether widely used as viscosity enhancer in eye drops, gelling agent in ocular injections and polymeric matrix in films, filaments and insert. HPMC is naturally uncharged, and has advantages of good biocompatibility, high solubility in water and thermoplastic behavior.[67] HEC (Natrosol™ 250) is a non-ionic, water-soluble hydroxyethyl cellulose. It is a versatile pharmaceutical excipient that can function as tablet-binder, film-coater or viscosity modifier.[68] Application of viscosity enhancers such as HPMC and HEC have been proved useful for improving the residence time of ocular formulations.[69, 70]

Plasticizer is another important component in the formulation of insert. Polyethylene glycol (PEG) is one of the frequently used hydrophilic plasticizer, and it is indispensable for development of film-based drug delivery as it could improve the mechanical properties of film, such as flexibility and fragility. [71] In our study, we selected PEG 400 as the plasticizer for the formulations of insert.

3.2.1 Optimization of Polymer Composition to Sustain Moxifloxacin Release from Inserts

The effect of ratio between polymers on the release profile of inserts was studied first. FS30D and EPO was selected as model polymers for two reasons. First, FS30D is anionic while MOX is cationic at neutral pH around 7, thus they could potentially form conjugates via

electrostatic interactions, which might prevent immediate release of MOX. Second, EPO and FS30D are counter-charged, and could also form a matrix wrapping MOX inside via electrostatic interactions, further prolonging the release time of inserts.[72] Three ratios of FS30D and EPO were tested, 80%-20%, 60%-40% and 40%-60%. Inserts prepared without FS30D were used as control. Formulation details are listed in **Table 6**.

Table 6 Formulation of MOX loaded inserts

Formulation	FS30D– EPO 80%-20%	FS30D– EPO 60%-40%	FS30D– EPO 40%-60%	EPO	FS30D–EPO 80%-20% HA
EUDRAGIT® FS 30 D	4.27 g	3.2 g	2.13 g	—	4.27 g
EUDRAGIT® E PO	0.32 g	0.64 g	0.96 g	1.28 g	0.32 g
HPMC K4M	0.2 g	0.2 g	0.2 g	0.2 g	0.2 g
HEC Natrosol™	0.2 g	0.2 g	0.2 g	0.2 g	0.2 g
PEG 400	0.8 g	0.8 g	0.8 g	0.8 g	0.8 g
Hyaluronic Acid	—	—	—	—	50 mg
Moxifloxacin	50 mg	50 mg	50 mg	50 mg	50 mg
Water	Up to 20 g	Up to 20 g	Up to 20 g	Up to 20 g	Up to 20 g

In this study, we aimed to design a sustained release formulation that could continuously release MOX for 24 h, so drug release profile of each formulation was tested. Ten-mm inserts containing approximately 300 µg MOX was cut from big films and placed in vials with 1 ml PBS under shaking at 100 rpm to identify which formulation could sustain MOX release for the longest time. As **Figure 4** shows, increasing the proportion of FS30D sustained the drug release of inserts. EPO inserts showed a burst release. With 80% of MOX was released within 1 hour and 90% of drug was released in 2 hours. With addition of FS30D, FS30D-EPO 40%-60% insert had a moderately sustained release profile, in which 70% of drug was release in the first hour. However, both formulations released 90% of drug within 2 hours, and their cumulative release reached nearly 100% in 6 hours. As the ratio of FS30D increased, the release of MOX was further sustained.

Compared with EPO insert, FS30D-EPO 60%-40% insert and FS30D-EPO 80%-20% insert only released 40% to 50% of drug in the first hour, and 55% to 70% of drug in 2 hours. Overall, FS30D-EPO 80%-20% insert performed the best, which achieved a sustained release of 8 hours. Consequently, the best ratio of FS30D to EPO was determined to be 80% to 20%.

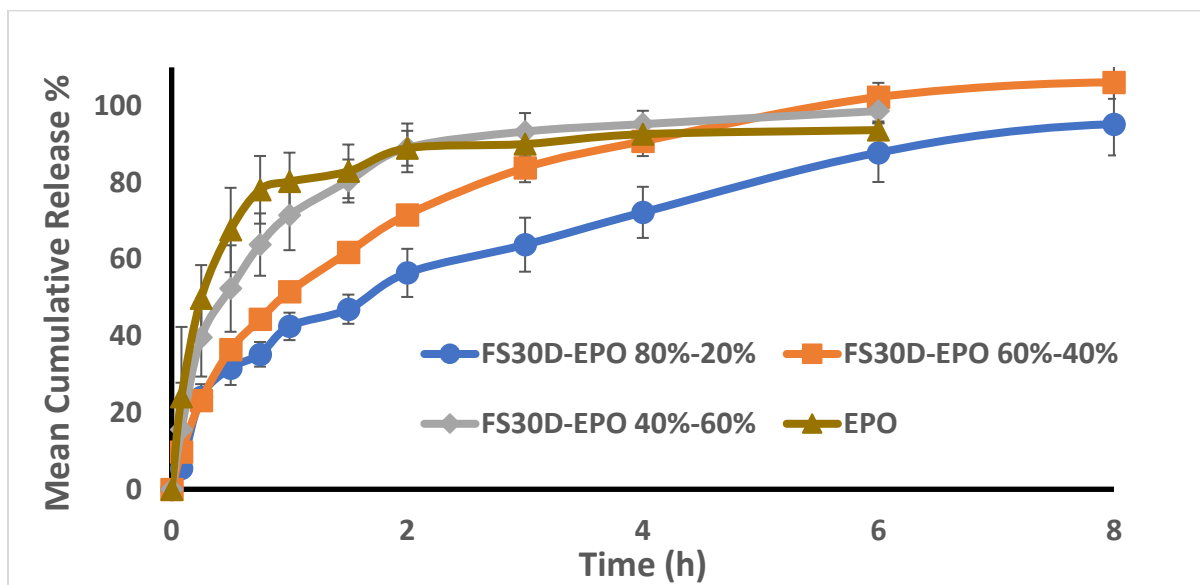


Figure 4 Drug release profile of MOX inserts prepared with different ratios of FS30D and EPO (n=3)

3.2.2 FS30D-EPO Combination Had the Best Sustained Release Profile Compared with FS30D-NM30D and FS30D-RS30D Combination

In addition to FS30D, other Eudragit[®] grades may also provide sustained drug release rate. NM30D and RS30D are two polymers that could be used for sustained release formulations. As mentioned previously, similar to EPO, these polymers are insoluble in aqueous solutions, and should be able to prevent disintegration of inserts in tear fluids. After setting 80% to 20% as the optimal ratio between FS30D and EPO, NM30D and RS30D replaced EPO in the formulation and

were tested for their ability to sustain MOX release. Compared with FS30D-EPO film, the ratio between FS30D and other polymers (NM30D/RS30D) was kept constant, but FS30D-NM30D and FS30D-RS30D films performed poorly in terms of drug release profile, as **Figure 5** shows both FS30D-NM30D and FS30D-RS30D films had a burst release in the first hour. Eighty-four percent and 72% of drug was released in 1 hour, 95% and 85% of drug was released in 2 hours, which were similar to the performance of EPO film. The results indicated that among all combinations, FS30D-EPO with a ratio of 80%-20% was the best option for sustained drug release, and it was selected as the formulation for subsequent experiments.

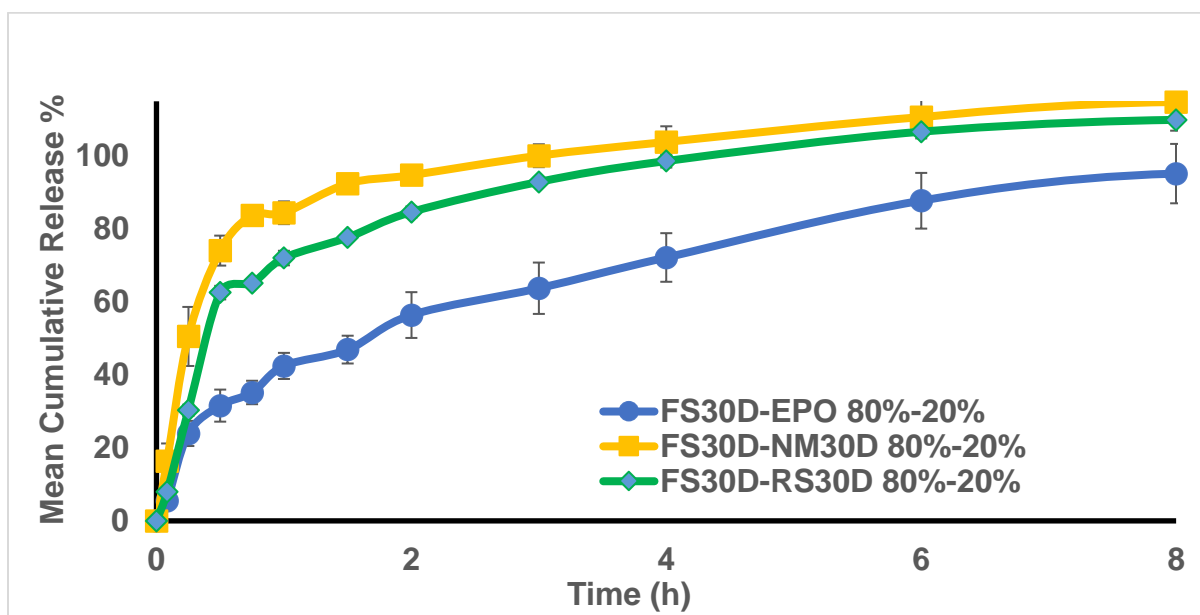


Figure 5 Drug release profile of FS30D-EPO, FS30D-NM30D and FS30D-RS30D inserts in ratio of 80%-20%(n=3)

Our result showed that the FS30D-EPO combination had the best performance in terms of drug release profile, and the release time was prolonged as the ratio of FS30D increased. However, the combination of FS30D-NM30D and FS30D-RS30D didn't achieve sustained release and showed a burst release in 1-2 hours. This could be attributed to lack of electrostatic reaction between polymers and drug. Moxifloxacin carries positive charge at neutral pH, and would form

conjugates with anionic FS30D, and cationic EPO would further react with FS30D and form the matrix.[72, 73] As RS30D and NM30D are non-ionic polymers, they might not be able to react with FS30D or moxifloxacin via electrostatic interactions, thus failed to retard the release of drug. Even so, there are still examples of successful fabrication of drug delivery systems with sustain release profile using RS30D and NM30D polymers, suggesting different combination of polymers are required to develop sustained release system for varying drugs.[74, 75]

Our target was to develop a MOX loaded insert that could sustain the release of drug for 24 h, and currently we achieved a sustained release of 8 h. However, the *in vitro* drug release study may not completely reflect the actual release profile *in vivo*. The tear volume of human is approximately 7 μ l, and the average tear turnover rate is 1.2 μ l/min [76, 77], suggesting that the total tear flow in human will be about 580 μ l in 8 h, and 1.73 ml in 24h, which is much lower than the volume of medium used in the *in vitro* studies. In our experiment, the volume of release medium was kept at 1 ml, and at each sampling time point, 400 μ l of fresh medium was replenished, making the total amount of medium used in the experiment approximately 4.4 ml for 8 h, and 4.8 ml for 24 h. However, 1 ml was the smallest possible amount used for *in vitro* drug release experiment because of the necessary volume needed for the hydration of inserts and sampling at each time point. Taking the volume difference between release medium and actual tear fluids, the drug release *in vivo* could turn out to be slower than it is in *in vitro* as tear fluids are much less, which may further limit the release rate of drug.

3.2.3 Increased Drug Loading Accelerated Drug Release Rate

For the following study of prophylactic efficacy of MOX loaded insert on rabbit endophthalmitis model, the drug loading of inserts had to be increased to 2.5 mg/insert to match the total dosage of MOX administered using Vigamox[®] (0.5% moxifloxacin, 10 drops, 50 µl per drop). To evaluate the effect of drug loading on release rate of MOX, the release profile of inserts with three drug loadings were tested. FS30D-EPO 80%-20% Films with MOX loading of 80 µg/5 mm insert, 1.25 mg/5 mm insert and 2.5 mg/5 mm insert were tested. As Figure 6 shows the initial drug release rate increased significantly with drug loading in the film. Compared with the film of 80 µg/5 mm insert drug loading, the one of 2.5 mg/5 mm insert drug loading exhibited a burst release of all its payload in 1 hour, while the one of 1.25 mg/5 mm insert had relatively faster release rate, but still maintained a sustained release profile over 8 hours. To prepare inserts with drug loading of 2.5 mg/5mm insert, 1.25 mg/5 mm insert and 80 µg/5 mm insert, 600 mg, 300 mg and 50 mg of MOX was used in the formulation for 20 ml of film-forming solution. The concentration of drug used to prepare film-forming solutions for 2.5 mg/5mm insert and 1.25 mg/5mm insert reached 30 mg/ml and 15 mg/ml, respectively, which was much higher than that in other sustained-release film formulations of moxifloxacin.[78, 79] As the concentration of MOX increased while that of FS30D and EPO remained unchanged, the amount of polymers could become insufficient and polymers might only be able to form matrix around a limited amount of MOX in the solution, leaving majority of MOX free and ready for immediate dissolution. Especially for 2.5 mg/5mm insert, the concentration of MOX in solution might have exceeded the solubility of MOX at room temperature, leading to drug precipitation, which could also be a reason for accelerated drug release.[57]

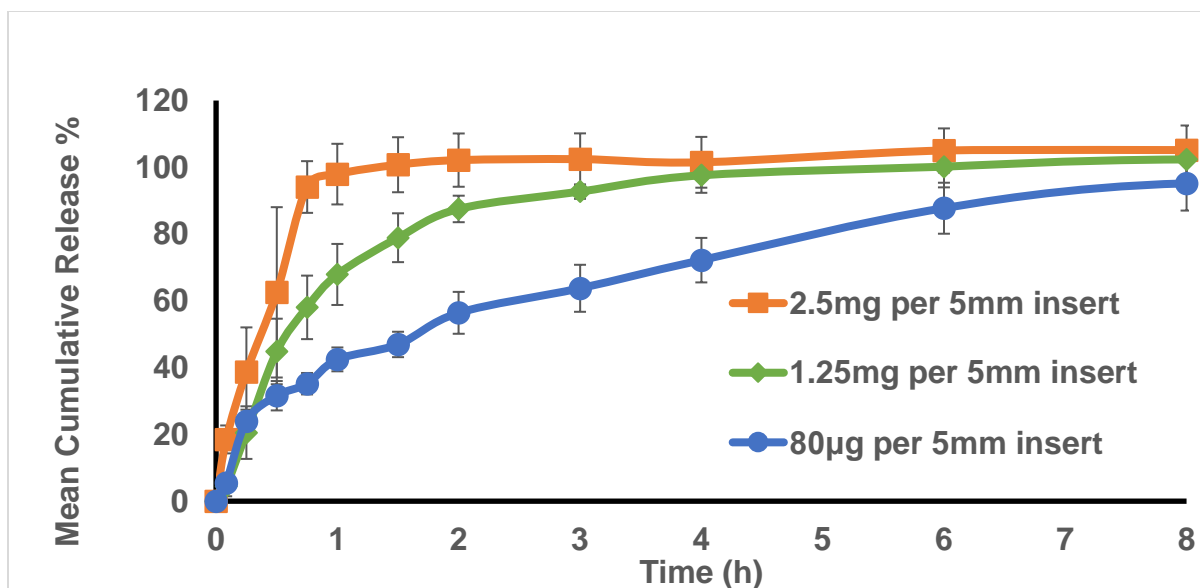


Figure 6 Drug release profile of FS30D-EPO 80%-20% inserts with different drug loading (n=3)

3.2.4 Incorporation of Hyaluronic Acid Did Not Alter the Release Profile of Insert

Hyaluronic acid (HA) may offer many beneficial effects in ophthalmic formulations. It is the main ingredient of artificial tear, which can reduce ocular irritation, moisturize eyes and replenish the deficiencies of sodium hyaluronate in the tear film; HA is also able to enhance muco-adhesion, promote wound healing and prevent harmful substances from binding to the eye; Additionally, HA has potential for sustaining the release of drugs. [80, 81]

An additional formulation of FS30D-EPO 80%-20% loading 50 mg MOX (80 µg/5 mm insert) supplemented with HA was tested to examine whether HA would influence the release profile of insert. As **Figure 7** shows, the release profile of film was almost identical to that of FS30D-EPO 80%-20% film prepared without HA.

The result indicated that HA didn't further extend drug release in our formulation, and incorporating HA in our formulation also caused problems in preparation of films such as drug

precipitation and high viscosity of film-forming solution affecting degassing process in solvent casting. While we attempted to increase drug loading in order to meet the need for equivalent dose with Vigamox[®] in endophthalmitis prophylaxis rabbit model (10 drops, 50 µl/drop, 2.5 mg moxifloxacin in total), the original sustained release profile of film was affected, and drug precipitated in the film. Drug precipitation deteriorated as HA significantly increased solution viscosity and affected the dissolution of drug. As such, HA had to be excluded from our formulation in spite of its potential benefits.

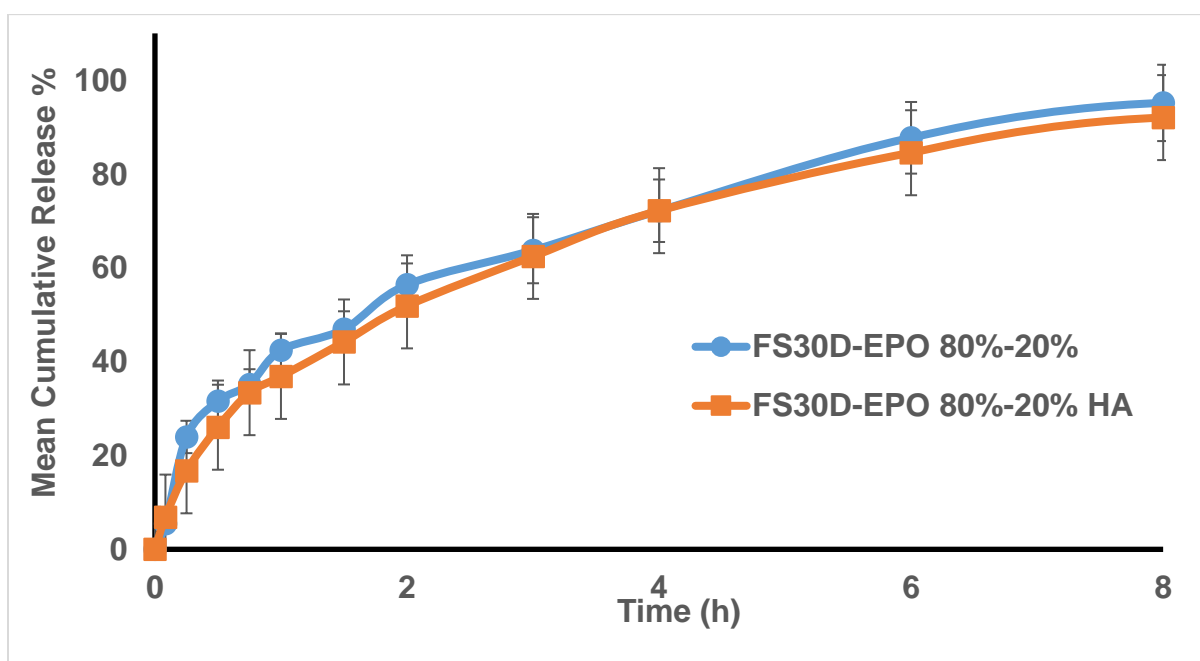


Figure 7 Drug release profile of FS30D-EPO and FS30D-EPO HA inserts (n=3)

3.2.5 Inserts Demonstrated Good Uniformity in Weight, Thickness and Drug Loading

Efficiency

The ophthalmic insert should have appropriate weight and thickness in order to retain in the eye without significant discomfort, and needs to have uniformity in terms of drug loading

efficiency to ensure the successful delivery of adequate drug upon insertion. The inserts were characterized, including weight, thickness and loading efficiency, and results are shown in **Table 7**.

The result showed that the film composition didn't significantly affect the weight or thickness of 10-mm inserts and the drug loading efficiency. With EPO replaced by NM30D and RS30D, the weight of disk remained between 22 to 25 mg, and the thickness of film was close to 0.3mm. While the loading efficiency of FS30D-RS30D remained at 80%, that of FS30D-NM30D decreased significantly to 65%, which might be caused by the formation of polymer aggregates during the preparation of solution. Extra HA in the formulation didn't significantly affect the characteristics of film including weight of disk, thickness of film and loading efficiency. In short, all formulations exhibited insert weight between 22 to 25 mg, and loading efficiency of approximately 80%, which achieved the target drug loading of 300 µg per 10 mm insert. The overall thickness (~0.3 mm) and weight of 10 mm disks (~23 mg) of our formulations was comparable with those of Ocusert™ (0.25-0.40 mm in thickness, ~22 mg in weight) and inserts developed by Pawar *et al.*[78, 82, 83]

Table 7 Characterization of inserts prepared with different formulations

Formulation	FS30D- EPO 80%-20%	FS30D- EPO 60%-40%	FS30D- EPO 40%-60%	EPO	FS30D- NM30D 80%-20%	FS30D- RS30D 80%-20%	FS30D- EPO 80%-20% HA
Weight per 10 mm insert (mg)±SD	22.70±1.41	23.90 ±1.10	24.60±0.64	22.67±0.06	24.33±1.69	21.80±1.83	24.98±4.59
Thickness (mm)±SD	0.29±0.023	0.31±0.018	0.31±0.010	0.31±0.030	0.31±0.023	0.30±0.024	0.27±0.028
MOX Loading Efficiency (%)±SD	80.76±0.33	81.38±1.99	80.33±2.05	80.74±2.16	65.56±6.32	82.14±15.34	80.74±4.88

3.2.6 Inserts Prepared Using Solvent Casting Method Exhibited Micropatterns with High Fidelity

The inserts were cut from film sheets prepared in micropatterned mold using solvent casting method. To examine the quality of micropatterns on films and determine if they could be further evaluated for their mucoadhesive ability in the future, the appearance of films was observed under microscope.

As **Figure 8** shows, the appearance of film changed with drug loading. While the FS30D-EPO 80%-20% film prepared with 50 mg moxifloxacin (300µg/10 mm insert) appeared slightly yellowish and transparent, with its patterned area visible, the film of same composition prepared

with 600 mg moxifloxacin (5 mg/10 mm insert) nearly lost its transparency, and the patterned area became totally invisible. However, the microscope images under 10X and 20X magnification (**Figure 4**) indicated that the micropatterned film was successfully fabricated with high fidelity using solvent casting method. Films with different micropattern shapes were fabricated using Polydimethylsiloxane (PDMS) mold, including circle, square and triangle in diameters of 100 and 200 μm , and spacing of 100 and 200 μm . **Figure 8C** explains the identification of micropatterns. All the micropatterns could be clearly observed and the edge of micropatterns was sharp and clean, exhibiting good quality.

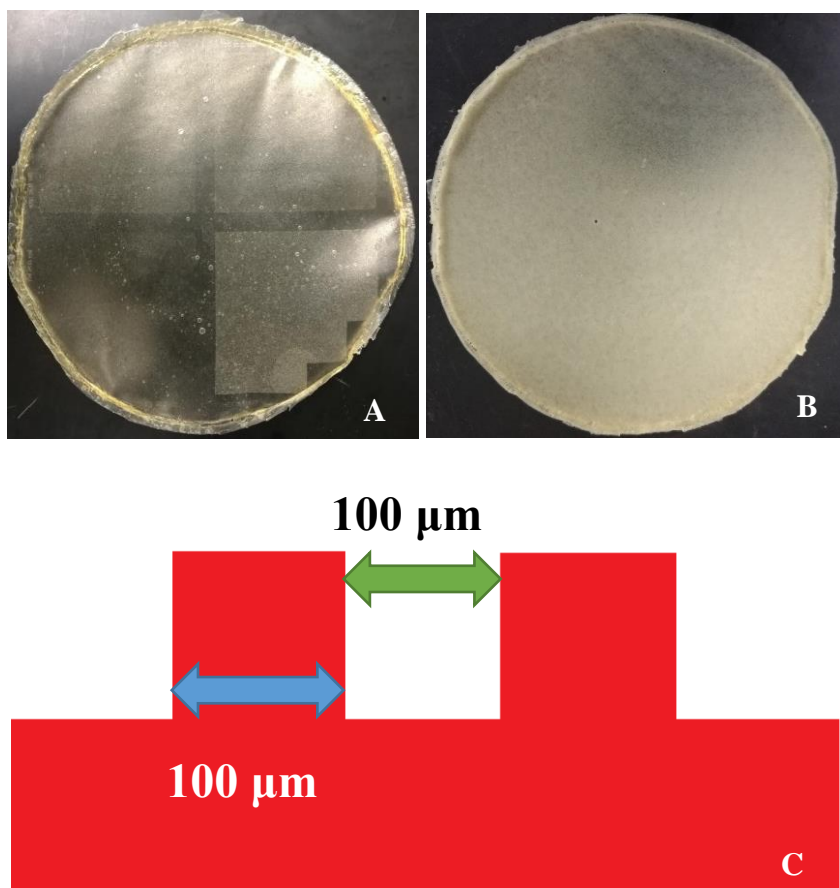


Figure 8 Photos of FS30D-EPO 80%-20% films and explanatory diagram of micropattern (A) Photo of micropatterned FS30D-EPO 80%-20% film loaded with 50 mg moxifloxacin. (B) Photo of micropatterned FS30D-EPO 80%-20% film loaded with 600 mg moxifloxacin. (C) Diagram explaining micropattern of 100 D100: The diameter of micropattern is 100 μm , and the distance between micropatterns is 100 μm

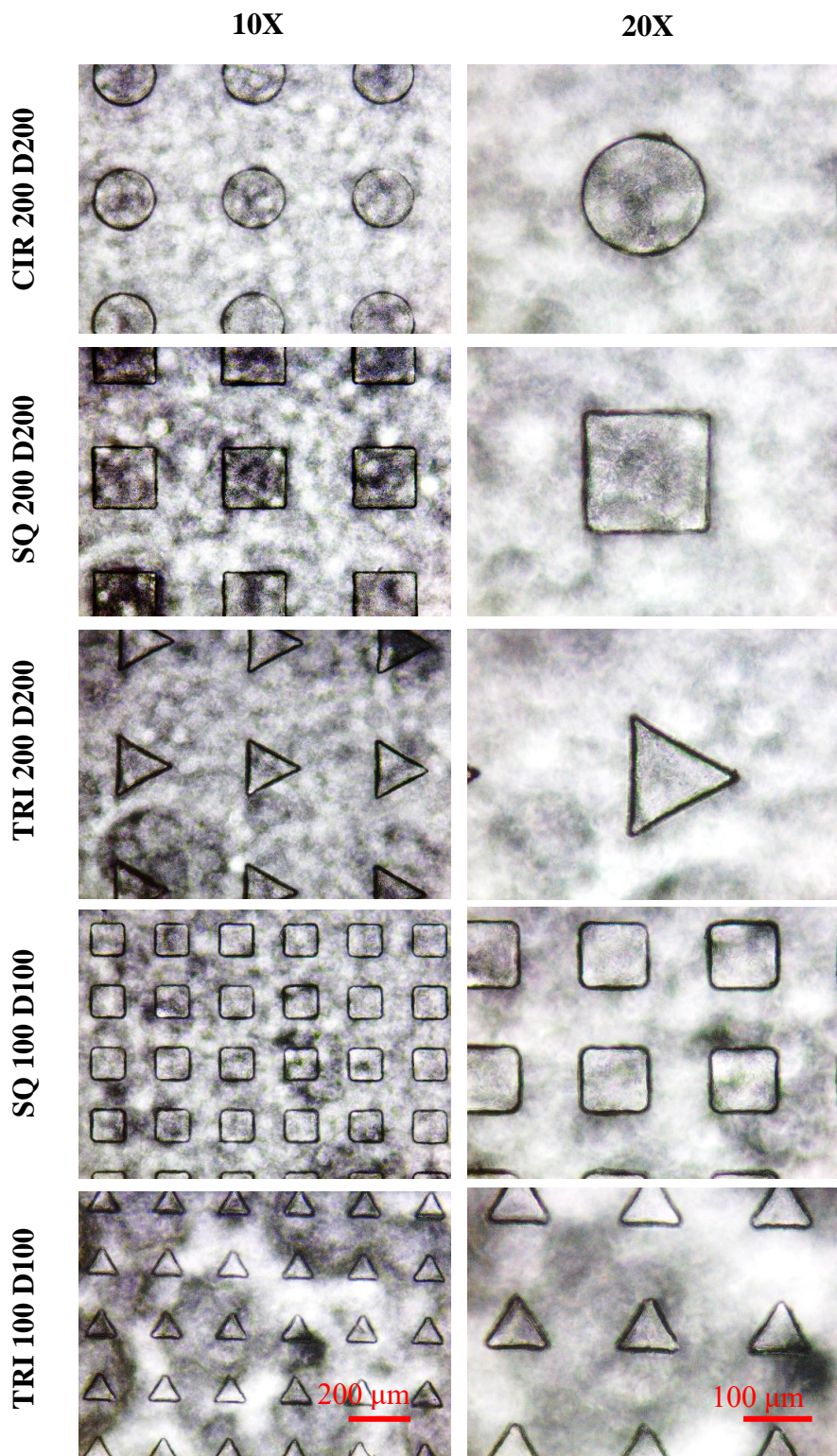


Figure 9 Micropatterns under microscope at 10X and 20X magnifications. CIR: Circle; SQ: Square; TRI: Triangle.

3.3 Validation of UPLC Method for Analysis of Moxifloxacin

3.3.1 Linearity

Linearity of detector response was established by plotting a regression curve of concentrations of moxifloxacin versus peak area. The linearity data are shown in **Table 8**, and the moxifloxacin calibration curve is shown in **Figure 10**. A representative chromatograph of moxifloxacin solution obtained by proposed method is shown in **Figure 11**, exhibiting a single sharp peak with a retention time of approximately 3.4 min. The result indicated satisfactory linearity between response and moxifloxacin concentration within the range of 1 to 200 µg/ml.

Table 8 Calibration table of UPLC analysis of moxifloxacin

Concentration (µg/ml)	Mean Peak Area	SD	RSD%
1	769	132	17.19
2.5	2723	230	8.47
5	6036	428	7.10
10	12727	1172	9.21
15	21024	1724	8.20
20	26120	167	0.64
25	32696	1461	4.47
50	68346	3804	5.57
100	134516	7531	5.60
150	189841	5764	3.04
200	240345	15727	6.54

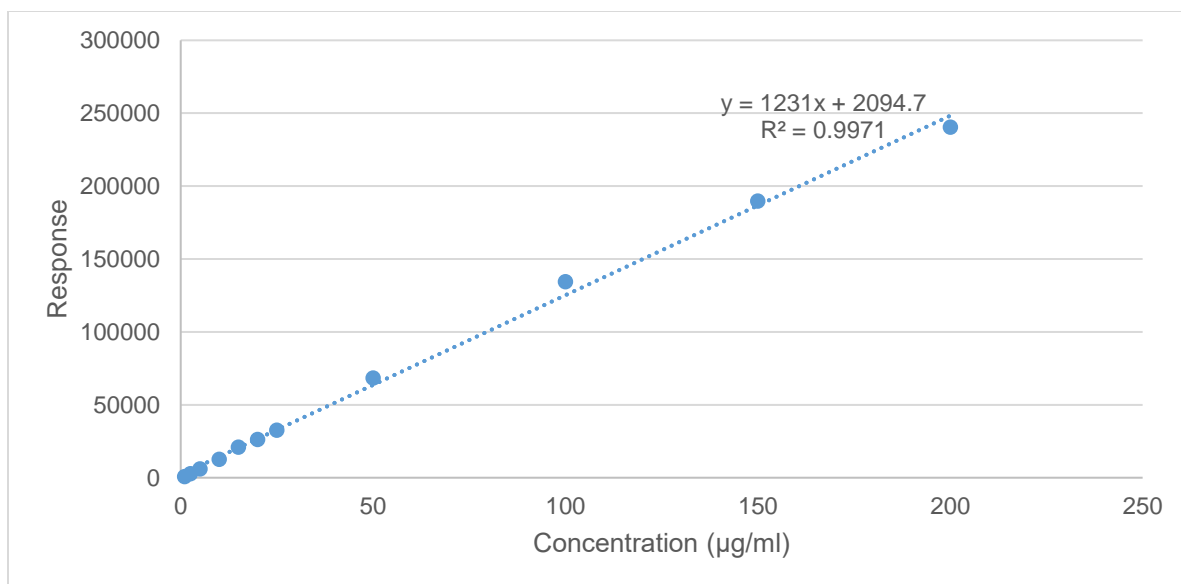


Figure 10 Calibration curve of moxifloxacin for UPLC

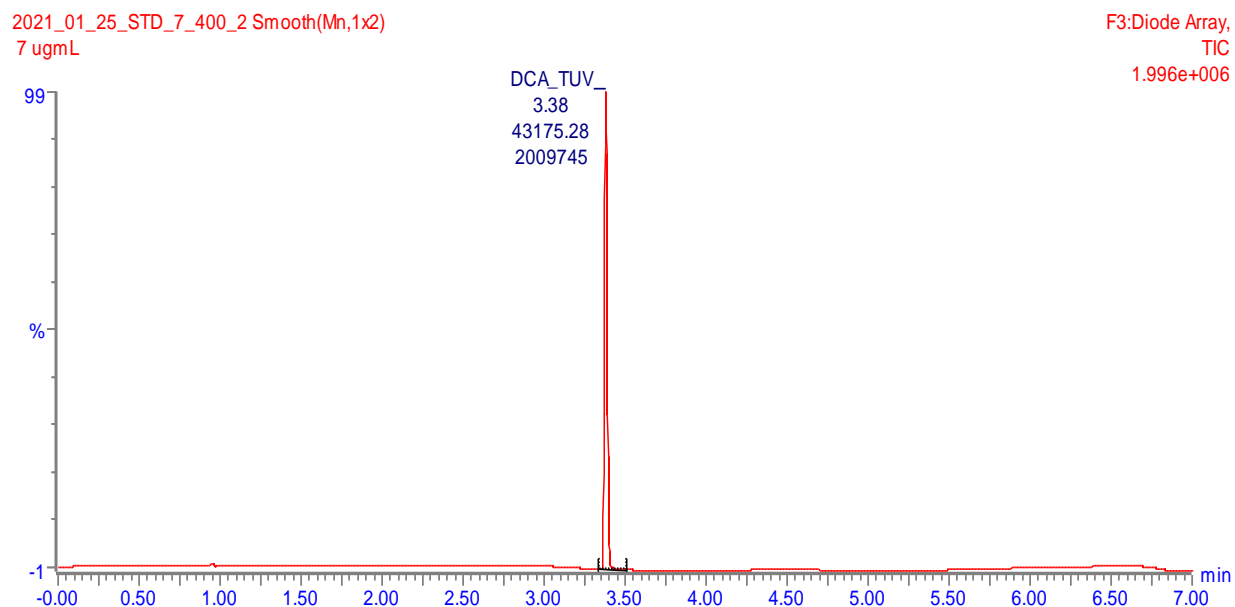


Figure 11 Representative chromatograph of moxifloxacin in UPLC

3.3.2 Accuracy

Accuracy of method was determined by recovery study. This accuracy study was conducted on 3 selected concentrations: 50 µg/ml, 100 µg/ml and 150 µg/ml, which were

approximately 50%, 100% and 150% level compared with the concentration of samples to be analyzed. Sample solutions of each level were prepared in triplicate and analyzed using method stated previously in this study. The mean peak area, mean calculated concentration, recovery %, standard deviation (SD) and relative standard deviation (RSD) % are listed in **Table 9**.

Table 9 Recovery study of moxifloxacin for UPLC method

Concentration (µg/ml)	Mean Peak Area	Mean Calculated Concentration (µg/ml)	Recovery%	SD	RSD%
50	66239	52.10	104.21	1.01	1.94
100	131778	105.93	105.93	5.49	5.18
150	188333	151.29	100.86	4.82	3.19

3.3.3 Precision

Intra-day and inter-day precision were determined as stated in the method section. Three concentrations within the linear range of calibration curve were selected for precision tests, 2 µg/ml, 80 µg/ml and 160 µg/ml, which also served as quality control at low concentration (QCL), quality control at medium concentration (QCM) and quality control at high concentration (QCH), respectively. The mean peak area, mean calculated concentration, deviation (DEV) %, SD and RSD % of intra-day (n=5) and inter-day (n=4) precision study are shown in **Table 10** and **Table 11**.

Table 10 Intra-day precision of UPLC analysis of moxifloxacin

Concentration (µg/ml)	Mean Peak Area	Mean Calculated Concentration (µg/ml)	DEV%	SD	RSD%
2	2483	1.90	-5.11	0.02	0.89
80	20488	80.49	0.61	0.40	0.49
160	398299	160.47	0.30	0.88	0.55

Table 11 Inter-day precision of UPLC analysis of moxifloxacin

Concentration (µg/ml)	Mean Peak Area	Mean Calculated Concentration (µg/ml)	DEV%	SD	RSD%
2	2233	1.92	-3.78	0.04	2.22
80	4540	84.82	6.02	3.64	4.29
160	10135	159.35	-0.41	4.77	2.99

3.3.4 Stability

Solution stability was tested on quality control samples stored at three conditions. One set of samples were kept at room temperature for 24 h, one set were kept at 4 °C in a refrigerator for 72 h, and one set was frozen and thawed for 3 times before test. The experiment was performed in triplicate. The mean peak area, mean calculated concentration, deviation (DEV) %, SD and RSD % of solution stability under different conditions are shown in **Table 12**.

Table 12 Solution stability of UPLC analysis of moxifloxacin

Concentration (µg/ml)	Mean Calculated Concentration ((µg/ml) ± SD (RSD%))		
	Room Temperature, 24 h	4°C, 72 h	Freeze/Thaw
2	1.71±0.03 (1.71)	1.68±0.01 (0.54)	1.83±0.01 (0.43)
80	85.31±1.15 (1.35)	84.63±0.23 (0.27)	84.24±0.40 (0.48)
160	158.43±3.83 (1.42)	158.21±3.46 (2.19)	159.56±2.28 (1.43)

Taking all results together, the proposed UPLC method was successfully validated and could be used for further analysis of MOX insert formulations.

3.4 Moxifloxacin Loaded Inserts Exhibited Good Stability under Different Storage Conditions

The stability of drug loaded inserts was evaluated by its weight and change in drug release profile. The weight of inserts measured at T0 and at the time of testing was compared, and the percentage variation in weight of inserts are shown in **Figure 12**. The weight of inserts stored at both 45°C/75% RH (Group 1) and 25°C/60% RH (Group 2) slightly increased by 0.1% and 0.36%, respectively, while that of inserts stored at 4°C in refrigerator (Group 3) remained almost unchanged (-0.02%) for the first 4 weeks. From week 4, more obvious weight variation of inserts in 45°C/75% RH Group and 25°C/60% RH Group could be observed. For 45°C/75% RH Group, the average weight of inserts increased by 0.75%, and for 25°C/60% RH Group, the increase was 3.33%. By week 10, the average weight of 45°C/75% RH Group increased by 1.36%. This test showed that the weight of film inserts remained relatively constant during the testing period under 40°C/75% RH, 25°C/60% RH and 4°C, suggesting little water was absorbed from external environment even in high humidity. Overall, the weight of inserts stayed stable during the test.

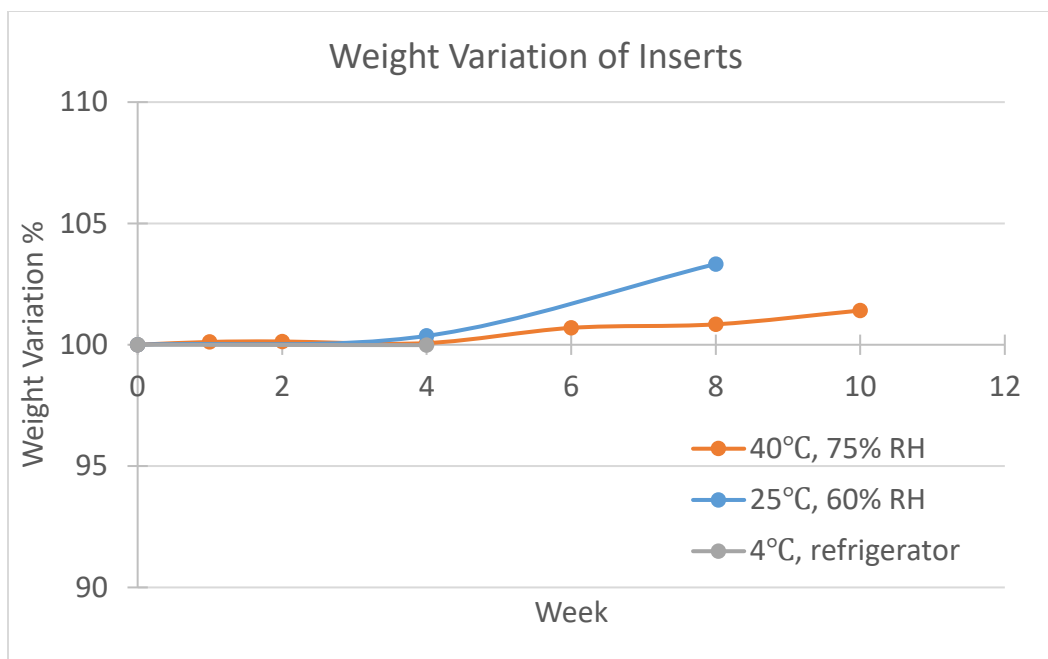


Figure 12 Weight variation of inserts stored under different conditions over time(n=9)

The drug release profile of inserts stored under various conditions was measured. As shown in **Figure 13**, inserts from 45°C/75% RH maintained almost identical drug release profile for the first 4 weeks. However, at week 6 and week 8, the drug release rate of inserts was faster for first 8 hours compared with that on earlier time points. Accelerated drug release rate in the first 6-8 hours was also observed among inserts from 25°C/60% RH Group and 4°C refrigerator Group. The cumulative drug release % at 4 hours and 8 hours increased by about 10% in both groups compared with their respective drug release profile of T0. However, despite some alteration in drug release profile, inserts from all groups were able to continuously release moxifloxacin for at least 6 to 8 hours without significant initial burst release.

Nonetheless, some interesting changes took place on the inserts. After storage, inserts kept under all conditions were more likely to stick to each other, and harder to remove from the surface of package. The texture of inserts also changed. Compared with fresh inserts, the stored inserts were stiffer and harder to bend, especially those kept under 4°C. Because of the limited number of

available samples for analysis, and this finding should be further quantified in the future by using equipment such as force gauge. This change could be a result of the crystallization of components in the formulation, which needs to be further validated by microscopy in the future. The formation of crystals might lead to a change in the structure of the film, thus influence drug dissolution from the film, which provided a possible explanation for the alteration of drug release profile.[84, 85]

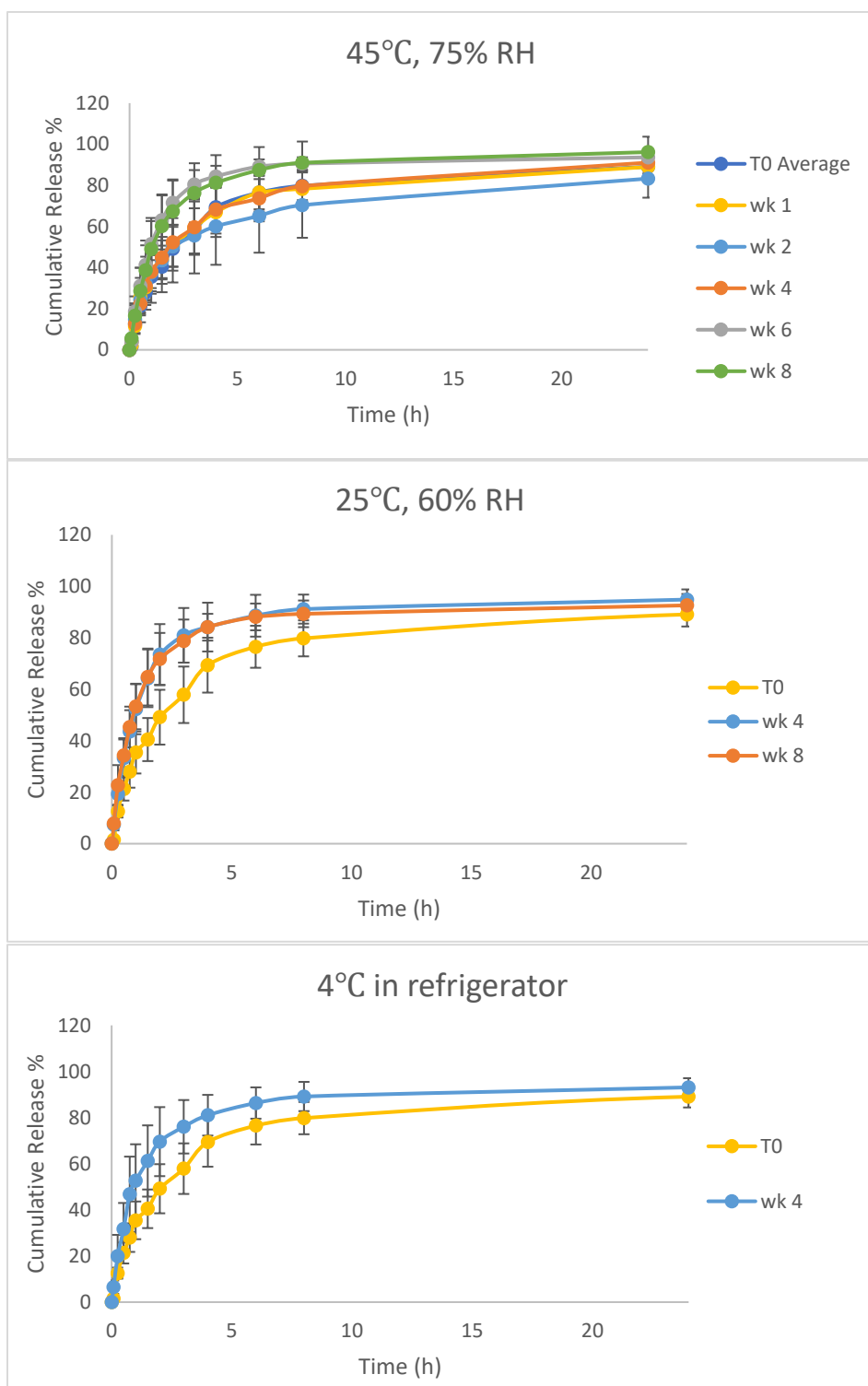


Figure 13 Drug release profile of inserts stored under different conditions over time (n=9)

3.5 Moxifloxacin Loaded in Inserts Remained Active Against Bacteria In Vitro After Preparation Procedures

Preparation of moxifloxacin loaded inserts using solvent casting method included one step of oven drying at 70-75°C, and there was risk that moxifloxacin might lose its anti-bacterial activity at high temperature during the process, which made it necessary to validate the activity of MOX loaded in inserts. The activity of MOX loaded in inserts was tested using Kirby-Bauer test, and the result is shown in **Figure 14** and **Table 13**. Based on the zone of inhibition, MOX release from inserts was calculated from the regression curve. The amount of active MOX released from both 5-μg and 8-μg inserts on *S. aureus* plates were in a range from 2.93 to 3.03 μg, and the amount of active MOX released from both inserts on *E. coli* plates were in a range from 2.26 to 3.85 μg. Despite different drug loading of inserts, both 5-μg and 8-μg inserts released approximately 3 μg of active MOX into plates seeded with *S. aureus* and *E. coli*. The result indicated that, although the amount of MOX released was incomplete, MOX in the inserts still remained active against bacterial after fabrication procedure.

Moxifloxacin can remain stable under 105°C for at least 24 hours, and the result indicating that MOX loaded in the inserts only partially kept its anti-bacterial activity could be caused by the incomplete release of MOX from inserts. [45, 86]. Compared with liquid medium used for *in vitro* drug release tests, agar plate is much less aqueous. On agar plate, the deficiency of aqueous environment might have prevented adequate hydration of the inserts, and limited drug dissolution from the inserts.

Table 13 Sizes of zone of inhibition on *S. aureus* and *E. coli* agar plates

MOX. (µg)	Mean Zone of Inhibition (mm) <i>S. aureus</i>		Mean Zone of Inhibition (mm) <i>E. coli</i>	
	Reader #1	Reader #2	Reader #1	Reader #2
12.5	30	29.5	28	29.5
5	27.5	27	26	26.5
4	27	27	25.5	26
3	26.5	26.5	25	26
2	24	24	24	25
1	21.5	22	23	24
0.5	20	20	21.5	23
0.1	12	11	15.5	16
Insert – 5 µg	25	25	25	25
Insert – 8 µg	25	25	25	25

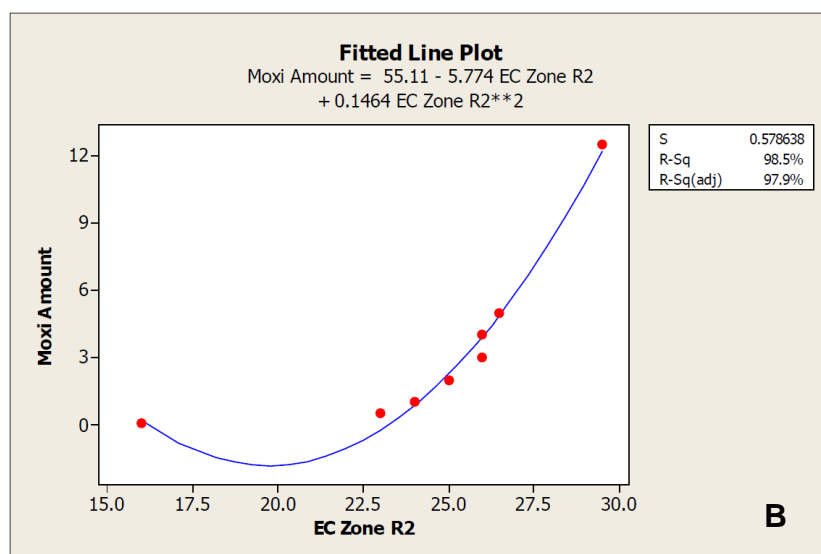
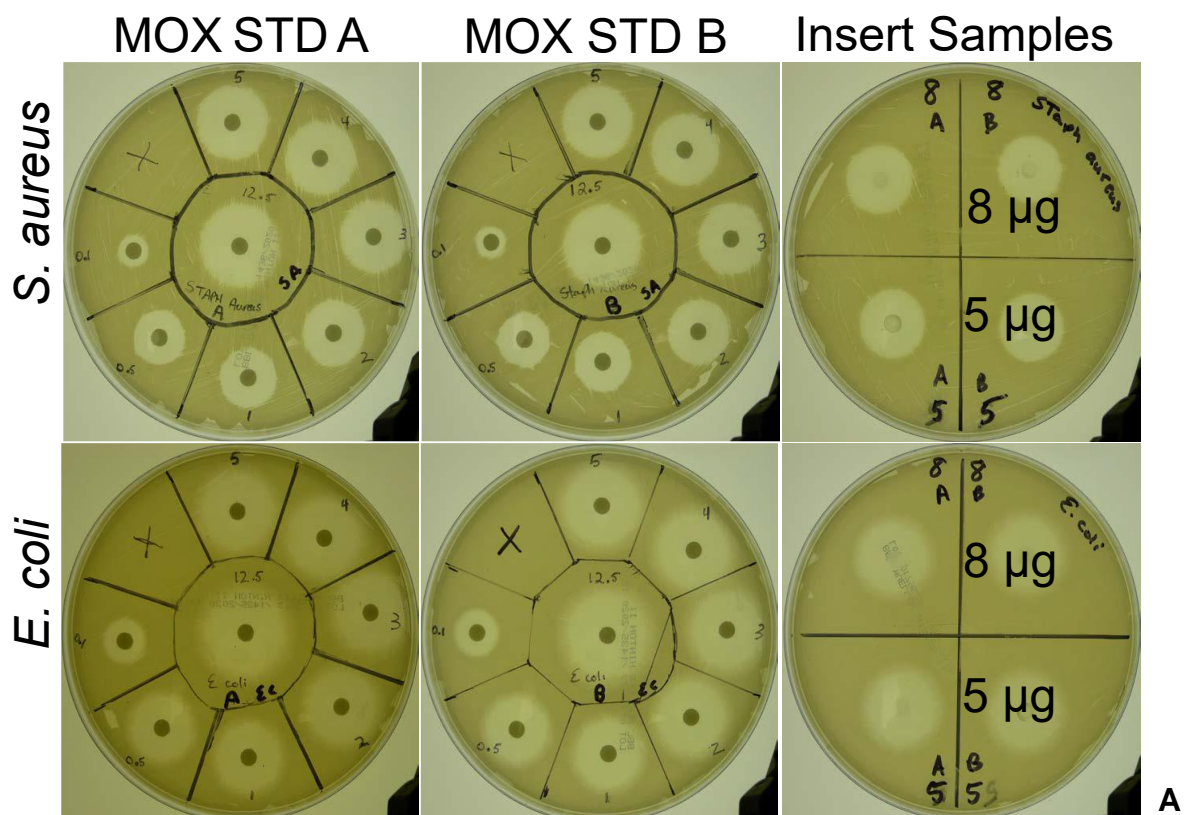


Figure 14 Validation of anti-bacterial activity in vitro. (A) Agar plates at 24 h after seeding. Paper disk instilled with MOX standard solution was placed in each section, which contained 0.1, 0.5, 1, 2, 3, 4 and 5 μ g of MOX (counterclock-wise); insert containing 12.5 μ g of MOX was placed at the center. **(B)** Representative regression curve of amount of MOX vs. zone of inhibition.

3.6 FS30D-EPO 80%-20% Insert Was Non-toxic to HCLE Cells *In Vitro*

The epithelium of ocular surface is an essential barrier to the toxic substances attempting to penetrate the cornea. Corneal epithelia cells can proliferate and rebuild ocular tissues, promoting corneal wound healing.[87] Corneal epithelium is a tissue constantly in fast regeneration, and a specialized stem cell population known as limbal cells are responsible for maintaining its integrity and functionality. [88] Human Corneal Limbal Epithelium (HCLE) cell is immortal cell line derived from those cells, and they were suitable subjects for cytotoxicity test since ocular epithelium would be in contact with film inserts directly. [53] Three formulations, FS30D-EPO 80%-20%, FS30D-EPO 40%-60% and EPO films, were tested. As **Figure 15** shows, all cells were able to attach to the plastic surface of 24-well tissue culture plate and form a confluent monolayer at the bottom of wells, while remaining their normal morphological characteristics that they demonstrated under normal culture conditions in flasks or plates, as compared with literatures.[53, 54, 89] The cells were then exposed to treatment with the film inserts for 24 h before being tested for viability via resazurin assay.

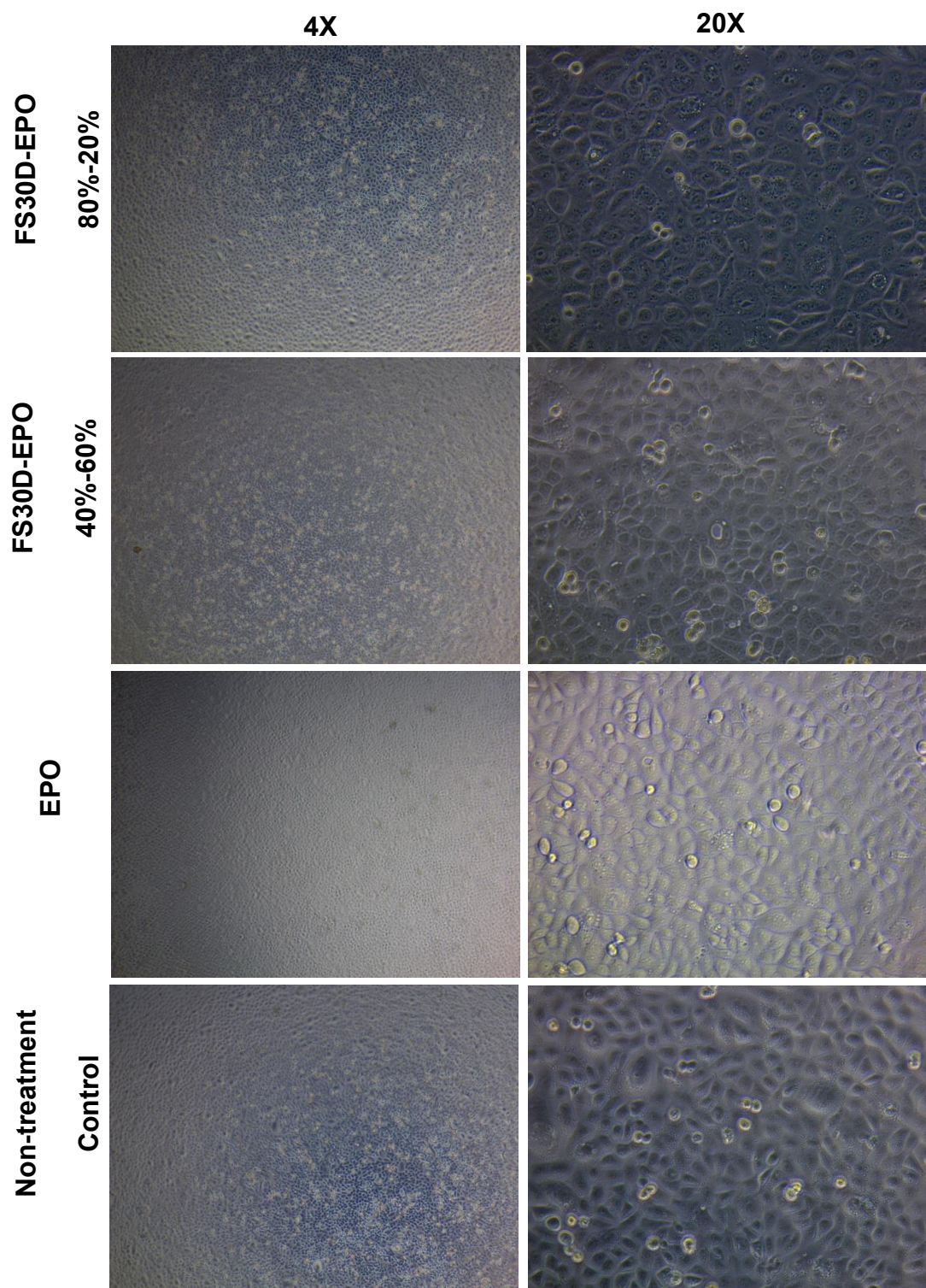


Figure 15 Representative microscopic images of HCLE cells in each group at 4X and 20X magnification pre-treatment.

The resazurin, also known as Alamar Blue, is a blue dye that can be internalized by cells.[90] Living mammalian cells, because of their metabolic activity, are able to irreversibly reduce resazurin via mitochondrial reductase, to resorufin, which is a highly fluorescent pink compound and will be released freely from cells after metabolization.[91] Measurement of cell viability using resazurin assay is based on the principle that the relationship between fluorescence output of resorufin and quantity of viable cells is proportional in a wide concentration range. [92]

Microscopic images of HCLE cells post-treatment are shown in **Figure 16**. Cells in groups treated with FS30D-EPO 80%-20% and FS30D-EPO 40%-60% films maintained their morphology, despite a few particles and debris from inserts observed in the background. As for cells treated with EPO film, some had already detached and lifted from the bottom of well. The cells that still remained adhered were partially deformed and lost their original morphology, transforming to crystal-like shape and releasing a large number of cell debris. The cells in non-treatment control group stayed mostly unchanged in terms of morphology. The cell boundary and tight cell junctures remained clearly visible throughout the experiment. In short, by comparing microscopic images of both pre-treatment cells and post-treatment cells, it could be concluded that FS30D-EPO 80%-20% and FS30D-EPO 40%-60% films were well-tolerated by HCLE cells, while EPO film exhibited considerable toxicity towards the cells.

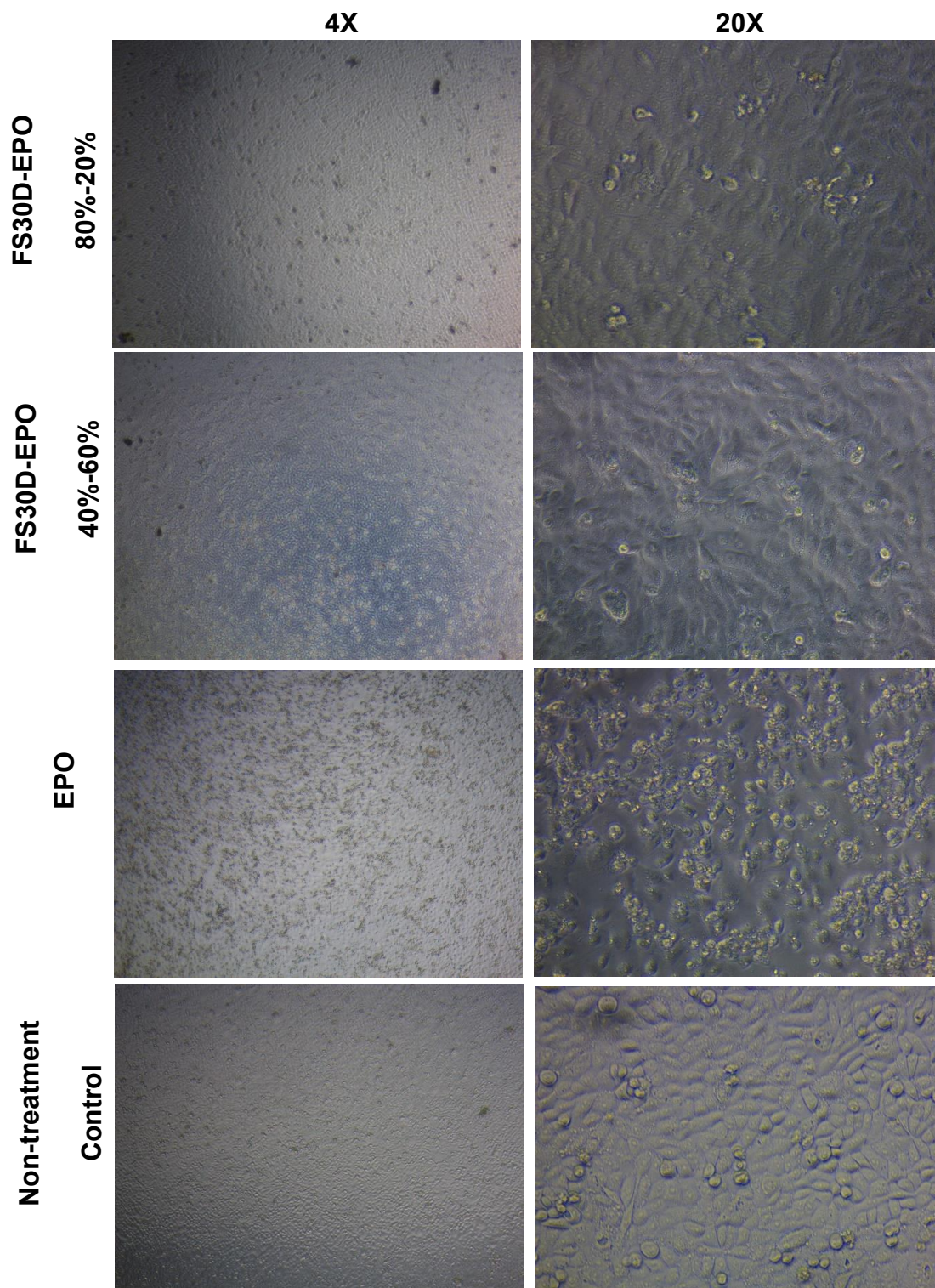


Figure 16 Representative microscopic images of HCLE cells in each group at 4X and 20X magnification post-treatment.

Results of resazurin assay on HCLE cells further confirmed that both FS30D-EPO films were non-toxic and EPO film affected cell viability significantly. As **Figure 17** shows, in two separate tests, cell viability in FS30D-EPO 80%-20% and FS30D-EPO 40%-60% film treated group remained close to 100%, comparable with non-treatment control group, whereas the viability of cell dropped to 40 to 47%, indicating significant toxicity.

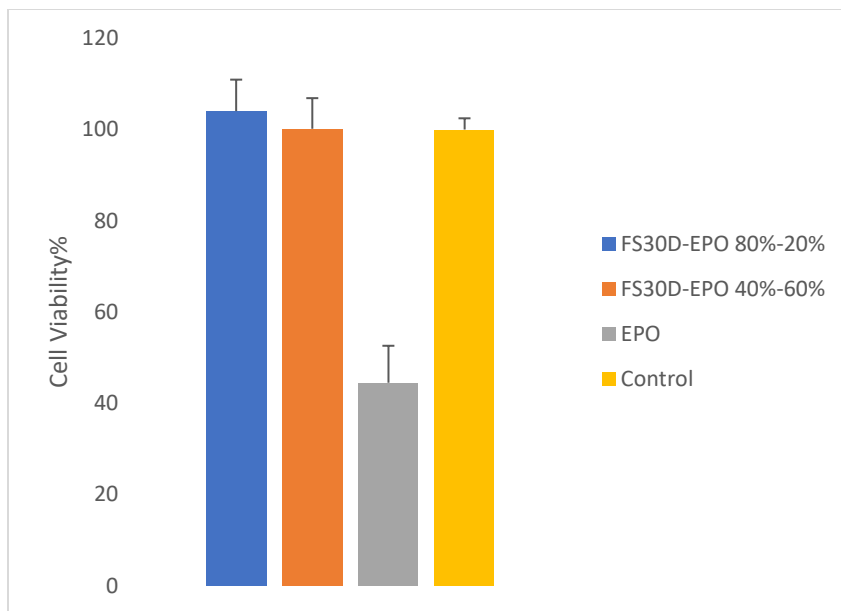


Figure 17 Cell viability of treatment groups and non-treatment control determined by resazurin assay. Results were from two independent experiments performed on cells revived from different cryopreservation vials and on different days. (n=5)

Our result demonstrated that formulations with FS30D-EPO ratio of 80%-20% and 40%-60% were non-toxic to the cells, but pure EPO formulation appeared to be significantly toxic. All polymers used in the formulation are categorized as GRAS by the FDA and have been approved as additives to pharmaceutical and food products.[65, 68, 93] Several studies examined the toxicity of dosage forms incorporating Eudragit® E polymers using multiple cell lines, but few reported the cytotoxicity of polymers.[94-97] This could be due to difference in the amount of Eudragit® E polymers used in the formulation. Instead of a few micrograms, the amount of EPO presented in

insert of 5 mm diameter would be milligram-level. From the microscope image, a large amount of particulate matter was found in the wells of EPO group, which could be precipitated EPO aggregates formed as inserts hydrated and partially lost integrity.

3.7 Evaluation of Prophylactic Efficacy of MOX Loaded Inserts *In Vivo*

3.7.1 Inserts Were Able to Retain in Taped-Shut Rabbit Eyes with Minimal Irritation

Before commencing the study on prophylactic efficacy of MOX loaded inserts, the experimental methods needed to be optimized to ensure the retention of inserts in rabbit eyes throughout the experiment. Five-mm inserts were used in the following tests exploring the experimental conditions for efficacy study.

The first test was to determine whether 5 mm inserts would stay in rabbit eyes overnight after placing them into the cul-de-sacs. As the result shown in **Table 13**, out of 4 inserts in unpatterned group, only 2 inserts retained for 6 hours, and 1 insert retained for 24 hours. For the inserts in other groups, only 1 insert in each group retained for 6 hours, and all inserts were removed or displaced before 24 hours. These results indicated that the inserts were unlikely to remain in place for sufficient time if the rabbits were allowed to blink their eyes and move freely after the insertion, regardless of the pattern of inserts. Measures had to be taken to prevent inserts from displacing during the experiment. The level of irritation was also examined by a clinician using Draize Scale (**Figure 18**)[55, 98], and the test result was also shown in **Table 13**, which demonstrated that the insert was non-irritative to the eye.

Table 14 Summary of preliminary insert retention test

Group	Total Insert Number	Number of Inserts Retained				Irritation Grading	
		2 hours	4 hours	6 hours	24 hours	6 hours	24 hours
Unpatterned	4	3	2	2	1	1.0 - PN Practically Non- Irritating	0.0 - N Non- Irritating
Square 100 D100	4	1	1	1	0	0.5 - N Non- Irritating	0.5 - N Non- Irritating
Triangle 100 D100	4	1	1	1	0	0.5 - N Non- Irritating	0.5 - N Non- Irritating
Square 200 D200	3	1	1	1	0	0.67 - PN Practically Non- Irritating	0.67 - PN Practically Non- Irritating

DRAIZE SCALE FOR SCORING OCULAR LESIONS

1. Cornea

A. Opacity-degree of density (area most dense taken for reading)	
No Opacity.....	0
Scattered or diffuse area, details "of iris clearly visible	1
Easily discernible translucent areas, details of iris slightly obscured	2
Opalescent areas, no details of iris visible, size of pupil barely discernible	3
Opaque, iris invisible	4
B. Area of cornea involved	
One quarter (or less) but not zero	1
Greater than one quarter, but less than half	2
Greater than half. but less than three quarters	3
Greater than three quarters, up to whole area	4
A X B X 5	Total Maximum = 80

2. Iris

A Values	
Normal	0
Folds above normal, congestion, swelling, circumcorneal injection (any or all of these or combination of any thereof) iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, hemorrhage, gross destruction (any or all of these)	2
A X 5	Total Maximum =10

3. Conjunctivae

A. Redness (refers to palpebral and bulbar conjunctivas excluding cornea and iris)	
Vessels normal	0
Vessels definitely injected above normal	1
More diffuse, deeper crimson red, individual vessels not easily discernible	2
Diffuse beefy red	3
B. Chemosis	
No swelling	0
Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of lids	2
Swelling with lids about half-closed	3
Swelling with lids about half-closed to completely closed	4
C. Discharge	
No discharge	0
Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to lids	2
Discharge with moistening of the lids and hairs, and considerable area around the eye	3
Score (A + B + C) X 2	Total Maximum = 20

Total Maximum Score: 110 represents the sum of all scores obtained for the cornea, iris and conjunctivae

I. A - Cornea Opacity

I. B - Cornea Area

I. (A x B) x 5 Cornea Total Score (Maximum Score = 80)

II. A - Iris Values

II. A x 5 - Iris Total Score (Maximum Score = 10)

III. A - Conjunctival Hyperemia

III. B - Conjunctival Chemosis

III. C - Conjunctival Discharge

III. (A + B + C) x 2 - Conjunctival Total Score (Maximum Score = 20)

Total Draize Score = I Tot. + II Tot. + III Tot. (Maximum Score = 110)

Maximum Mean Total Score = MMTS

Classification of Eye Irritation Scores

MMTS	Classification	Symbol
0.0 – 0.5	Non-Irritating	N
0.6 – 2.5	Practically Non-Irritating	PN
2.6 - 15.0	Minimally Irritating	M ₁
15.1 - 25.0	Mildly Irritating	M ₂
25.1 - 50.0	Moderately Irritating	M ₃
50.1 - 80.0	Severely Irritating	S
80.1 - 100.0	Extremely Irritating	E
100.1 - 110.0	Maximally Irritating	M _x

Figure 18 Draize Scale of irritation test

A subsequent test was performed to determine whether 5 mm film inserts would stay in rabbit eyes overnight after placing them into the cul-de-sacs and taping the eyes shut, and whether pre-wetting the inserts would improve retention in eyes left naturally open. In this experiment, only one type of patterned insert (Square 100 D100) was tested together with unpatterned insert. As **Table 14** shows, all inserts remained in taped shut eyes for 24 hours except for one, while none of the inserts in open eyes retained for 3.5 hours. The result suggested that taping the eye shut would effectively keep inserts staying in the eye, but pre-wetting the inserts would not improve the retention of neither patterned nor unpatterned inserts with eyes left open. The level of irritation was also examined, and result indicated that inserts were slightly more irritative as they retained in the eye for longer time. However, despite a minor increase in scores, the inserts were graded as minimally irritating, which was consistent with the result from first test.

Table 15 Summary of insert retention in taped and un-taped rabbit eyes

Group	Total Insert Number	Number of Inserts Retained			Irritation Grading
		3.5 hours	5.5 hours	24 hours	24 hours
Unpatterned-Taped	4	4	4	4	5.0 – M ₁ Minimally Irritating
Unpatterned-Not taped	3	0	0	0	0.0 – N Non-Irritating
Square 100 D100-Taped	4	4	4	3	3.0 – M ₁ Minimally Irritating
Square 100 D100 Not Taped	4	0	0	0	0.0 – N Non-Irritating

In the preliminary experiments prior to efficacy study *in vivo*, one major issue emerged was the inability of inserts to retain in place after insertion, which was observed on 5 mm inserts in this study, regardless of their micropatterns. Theoretically, micropatterns would be able to improve the adhesion and friction of devices on mucus-rich surfaces such as intestines.[99] In the earlier studies conducted by our group, we found that micropatterns could improve residence time of inserts in an *ex vivo* model using porcine eyes (unpublished data). Our *in vivo* result showing that neither patterned nor unpatterned inserts couldn't retain in the open eye of rabbits was not consistent with our previous *ex vivo* result. However, it could be due to a few reasons.

Porcine eye is often used for *ex vivo* models in vision sciences research as it's quite similar to human eye in many perspectives such as morphology, anatomical structure and reaction to inflammation, etc.[100] However, rabbit eye is much more different from human eye in terms of many parameters including anterior-posterior length, equatorial diameter, white-to-white measurements, anterior chamber diameter, ciliary sulcus diameter, and crystalline lens diameter and thickness, which may considerably affect experiment result if the differences are not taken into account for correct interpretation, especially in studies involving tests of sizing and fitting of ocular drug delivery systems or devices.[101-103] In our case, the nictitating membrane of rabbit eyes could be a crucial factor preventing inserts from staying in the cul-de-sac of rabbit, as it would squeeze insert out of the place when rabbit blinked. In another study that examined the efficacy of moxifloxacin loaded microspheres in endophthalmitis prophylaxis using the same rabbit model, the retention problem of microspheres was avoided by removal of nictitating membrane prior to experiment.[104] Besides, the size and thickness might also affect the retention of inserts. In our preliminary *in vitro* studies, the diameter of inserts would often grow by 30-40% upon hydration

probably because of the swelling of polymers.[105-107] (Data not shown) As the inserts hydrated, the increase of size and thickness could result in more difficulties for them to remain in place.

3.7.2 MOX Loaded Inserts Showed Comparable Prophylactic Efficacy with Vigamox® on Endophthalmitis Rabbit Model

Based on these test results, we decided to tape rabbit eyes shut during the MOX efficacy study to ensure insert retention and achieve best performance for inserts. While 5 mm inserts were determined to be the optimal size for in vivo study, 8 mm inserts containing 2.5 mg moxifloxacin were selected for efficacy examination since they could more effectively carry equivalent amount of drug to the total dosage administered in endophthalmitis prophylaxis using Vigamox® eyedrops (0.5% moxifloxacin, 10 drops in total, 50 µl per drop). The development of Endophthalmitis was examined at 24 h post-inoculation by a masked clinician based on the criteria listed in **Table 15**, and any eye with a Anterior Chamber Score (AC) of 3.0 or more in the anterior chamber inflammation exams of Hypopyon, Iritis, Fibrin Production, AC Cell, and AC Flare (shaded areas in **Table 15**), with a positive bacterial culture or diagnosed with endophthalmitis by physician based on clinical judgement was considered to have endophthalmitis. Median total score (TS), AC score (AC) and conjunctival score (total of discharge, conjunctival/scleral injection and limbal injection, CONJ) of each group were tested for statistical significance using Kruskal-Wallis test and Dunn's multiple comparisons test.

Table 16 Summary of clinical endophthalmitis evaluation

0 = Normal; 0.5 = Trace; 1.0 = Mild; 1.5 = Mild/Moderate; 2.0 = Moderate;
2.5 = Moderate/Severe; 3.0 = Severe

Rabbit	Group	Dis-charge	Conj/Scleral Injection	Limbal Injection	Hypo-pyon	Iritis	AC Cell	AC Flare	Fibrin	Corneal Infiltrate	Total Score	AC Score	Physician Clinical Endophthalmitis + or -
1	MOX	0	0	0	0	0	0	0	0	0	0.0	0.0	–
2	MOX	0	0	0	0	0	0	0	0	0	0.0	0.0	–
3	MOX	1.0	1.0	1.0	0	0	0	0	0	0	3.0	0.0	–
4	MOX	0	1.0	1.0	0	0	0	0	0	0	2.0	0.0	–
5	MOX	1.0	1.0	1.0	0	0	0	0	0	0	3.0	0.0	–
6	MOX-FI	2.0	3.0	3.0	0	1.0	0	1.0	0	0	10.0	2.0	–
7	MOX-FI	2.0	2.0	2.0	0	0	1.0	1.0	0	0	8.0	2.0	–
8	MOX-FI	2.0	2.0	2.0	0	1.0	0	1.0	0	0	8.0	2.0	–
9	MOX-FI	2.0	1.0	1.0	1.0	0	0	0	0	0	5.0	1.0	–
10	MOX-FI	3.0	1.0	1.0	0	1.0	0	0	0	0	6.0	1.0	–
11	CON-FI	1.0	3.0	3.0	0	2.0	2.0	2.0	1.0	2.0	16.0	7.0	+/-
12	CON-FI	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	27.0	15.0	+
13	CON-FI	1.0	2.0	2.0	1.0	2.0	2.0	2.0	3.0	2.0	17.0	10.0	+
14	CON-FI	1.0	1.0	1.0	0	1.0	1.0	1.0	0	1.0	7.0	3.0	–
15	CON-FI	3.0	2.0	2.0	2.0	3.0	3.0	3.0	3.0	3.0	24.0	14.0	+

MOX: Vigamox[®] group; MOX-FI: Moxifloxacin insert group; CON-FI: Placebo insert group

In vivo efficacy result demonstrated that both prophylaxis method using Viagmox® and inserts could effectively prevent the development of endophthalmitis, as well as the growth of fluoroquinolone-susceptible *Staphylococcus aureus* in the ocular tissue. None of the eyes treated with eyedrop or inserts had positive bacterial culture or developed endophthalmitis, while 3 out of 5 eyes treated with placebo inserts were positive in bacterial culture, and all 5 untreated eyes developed endophthalmitis.

Kruskal-Wallis test of the three clinical scores indicated that there was significant difference among groups (TS, $p < 0.0001$; AC, $p < 0.0001$; CONJ, $p = 0.0052$). Dunn's multiple comparisons test of the clinical scores showed that in terms of prevention of endophthalmitis, Viagmox® could significantly reduce TS and AC compared with placebo inserts (TS, $p = 0.0026$; AC, $p = 0.0009$), and moxifloxacin inserts demonstrated equivalent efficacy to Viagmox® (TS, $p > 0.05$; AC, $p > 0.05$). In addition, the CONJ of MOX group was significantly lower than MOX-FI and CON-FI group ($p < 0.05$), while there was no significant difference between MOX-FI and CON-FI group. Combining above results together, it could be concluded that moxifloxacin insert was equally effective in preventing development of endophthalmitis in rabbit eyes, compared with prophylactic use of Viagmox®.

Table 17 Summary of *in vivo* efficacy study

Group	Number of Eyes with Clinical Endophthalmitis based on AC score	Number of Eyes with Clinical Endophthalmitis based on clinical judgement	Number of Eyes with Microbiological Endophthalmitis based on bacterial culture	Number of Eyes <u>without</u> Clinical Endophthalmitis but with a Positive Bacterial Culture	Number of Eyes with <u>Clinical or Microbiological Endophthalmitis</u>
MOX	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
MOX-FI	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
CON-FI	5/5 (100%)	4/5 (80%)	3/5 (60%)	0/0 (0%)	5/5 (100%)

MOX: treated with Viagmox®; MOX-FI: treated with moxifloxacin loaded insert; CON-FI: treated with placebo insert

Table 18 Summary of clinical evaluation scores

Group	Total Score Median (Range)	Anterior Chamber Score Median (Range)	Conjunctival Score Median (Range)
MOX	2 (0-3)	0 (0-0)	2 (0-3)
MOX-FI	8 (5-10)	2 (1-2)	6 (4-8)
CON-FI	17 (7-27)	10 (3-15)	7 (3-9)

MOX: treated with Vigamox®; MOX-FI: treated with moxifloxacin loaded insert; CON-FI: treated with placebo insert

The endophthalmitis prophylaxis rabbit model used in our study has been widely-used in many other publications.[104, 108-121] To establish endophthalmitis in rabbits, the selected CFU and volume of bacterial inoculum was 6.24×10^4 and 25 μ l in our experiments. The respective numbers are usually different in the literature. For example, Castro *et al.*[108] injected 25 μ l of bacterial inoculum containing 5×10^5 CFU of *Staphylococcus aureus* into anterior chamber to establish endophthalmitis, Ermis *et al.*[109, 110] inoculated 0.1 ml saline containing 1×10^5 CFU of *Staphylococcus aureus* and *Staphylococcus epidermidis* into the vitreous cavity, and Norcross *et al.*[119] used 50 μ l inoculum containing 1×10^6 CFU of *Streptococcus pneumoniae* injected into the aqueous humor for the same purpose. This difference in CFU numbers could be caused by the selection of inoculation site. Compared with cornea, anterior chamber, aqueous or vitreous humor are harsher and more unfavorable environment to proliferate and reproduce. In rabbits, there is high possibility that the bacteria are eradicated from ocular tissues within days post-inoculation, even if there is no treatment applied.[113] This type of spontaneous sterilization has been reported in several studies, and it is hypothesized to be related to a series of changes occurred of the vitreous body after the inoculation, such as the depletion of nutrients and oxygen, the accumulation of secretory materials, and the accumulation of antibacterial substances and inflammatory cells as the space is highly restricted in vitreous cavity.[113, 122, 123]

Cornea was the selected inoculation site in this study since it was easier to establish endophthalmitis by corneal inoculation using a relatively small number of bacteria but still in a consistent and reproducible pattern. In the past studies, it had been demonstrated that too few bacteria might not be able to induce endophthalmitis in some rabbits, while too many bacteria might cause severe reaction such as acute conjunctival inflammation, which could seriously interfere with following clinical examination and grading.[116]

Clinical scores are commonly used for evaluation of outcomes in studies regarding toxicity, irritation or efficacy of ophthalmic drugs, preparations and devices. Despite some variation in grading scale and exact items included, all score systems generally focus on conjunctiva, cornea, iris and anterior chamber, which usually develop symptoms first in case of ocular diseases.[124-128] The determination of endophthalmitis in our study was based on three standards: clinical scores, bacterial culture and clinical judgement of physician. Clinical scores were specifically categorized into anterior chamber score (AC), total score (TS) and conjunctival score (CONJ). In our study, AC was used as an indicator for the severity of intraocular inflammation related to endophthalmitis, as endophthalmitis was typically associated with typical signs of anterior-chamber cell and flare with fibrin and/or hypopyon, also known as toxic anterior-segment syndrome (TASS).[129] TS was also considered as a measurement of ocular inflammation. In contrast with AC, TS indicated severity of all signs associated with inflammatory reactions both intraocular and peri-ocular. Our result showed that in terms of AC and TS, there was no significant difference between MOX group and MOX-FI group, while the scores of CON-FI group were significantly higher than MOX group, suggesting that Vigamox® and moxifloxacin inserts had equivalent efficacy in preventing endophthalmitis. However, oddly enough, when comparing the AC and TS between MOX-FI group and CON-FI group, the significance of difference was absent

(AC, $p=0.2115$; TS, $p=0.5338$). This could be a result of the small group size ($n=5$) used in this study. In studies that adopted the same experimental approach, usually at least 10 rabbits were included in one group. A study tested the prophylactic potential of Vigamox® incorporating three regimens: pre-challenge prophylaxis, post-challenge prophylaxis and full prophylaxis. The same animal model and clinical grading system was used, and the TS of pre-challenge group in that study (7.00) was quite close to the TS of MOX-FI group in our study (8.00).[114] This may suggest that the prophylactic efficacy of moxifloxacin insert was comparable with Vigamox® administered prior to bacterial challenge. Notably, during the clinical evaluation, it also appeared that the insert might be able to prevent bacterial infection in the cornea since no corneal infiltrate was observed in either MOX group or MOX-FI group. Nevertheless, this finding still needs to be validated, and corneal tissues need to be collected for bacterial culture in the future experiments.

It was important to evaluate ocular irritation of inserts as they might need to stay in contact with ocular surface for longer time. CONJ indicated the reaction of conjunctiva to bacterial inoculation and treatment. Since the inserts were placed in the cul-de-sac of rabbits and rabbit would perceive them as foreign bodies, it could also be considered as an indicator for the safety of inserts as it reflected the level of irritation or inflammation caused by them. However, because the number of animals used in this study was limited, and there were not adequate animals to set up another non-treatment control group, we could not distinguish the inflammatory reaction induced by insert from that induced by infection. As such, CONJ alone might not distinguish if the inflammatory reaction was induced by the inserts. To address this concern, we implemented Draize Test, which has been used to evaluate the ocular irritation potential of chemicals and pharmaceutical preparations as an official model worldwide for several decades, and experimental procedures based on it are included in government regulations and guidelines such as Organization

for Economic Cooperation and Development (OECD) guidelines for testing chemicals for acute eye irritation/corrosion. [130, 131] In our preliminary tests of insert retention, we tested placebo inserts in healthy rabbit eyes, and demonstrated that the inserts were minimally irritating after 24 h post-insertion using the Draize Scale. Still, it should be admitted that although 3-6 subjects are typical group size for Draize Test, additional animals need to be included in the experiment to acquire more accurate and conclusive result.[131, 132]

4.0 Conclusion and Future Directions

In this study, we first came up with a moxifloxacin loaded ophthalmic insert that could be administered topically and continuously release the drug for up to 8 hours, which has the potential to significantly reduce the administration frequency of drug and improve patient compliance. This insert may be easily applied and removed by patient independently, and could possibly fill in the gap of lacking long-time delivery of antibiotics in current prophylactic methods of endophthalmitis.

In this study, we successfully fabricated and characterized a micropatterned moxifloxacin loaded film, and validated a UPLC method that could be implemented in subsequent analysis of the formulation. *In vitro*, we showed the activity of moxifloxacin loaded in inserts, and the formulations composed of Eudragit® FS30D and EPO were found to be non-toxic to HCLE cells. *In vivo*, we showed “proof of principle” study in endophthalmitis rabbit model, demonstrating moxifloxacin loaded inserts had comparable efficacy with Vigamox® eyedrop in prophylaxis of endophthalmitis. However, this study had its limitations as well. The insert characterization didn’t include tests on mechanical properties, the stability tests only provided data for up to 12 weeks and did not assess the degradation of moxifloxacin in inserts, the study was relatively small and some results were not conclusive, and the inserts used for animal study was not sterilized as per clinical practice.

In the future, we will include mechanical strength tests in characterization of inserts, such as tensile strength test and Young’s modulus test, and further optimize the UPLC method so that it can be used for analyzing drug degradation in formulation stability tests and to measure MOX concentration in the ocular tissues collected in animal study. The formulation of film will be further

optimized as well in order to reduce size and thickness while maintaining current drug loading. As micropatterned films, the mucoadhesion and retention of formulations will also need to be tested using *ex vivo* or *in vivo* approach. As for *in vivo* study, experiments with improved design involving larger number of subjects and appropriate control groups will be set up, different insert shapes, such as crescent shape that fits the edge of eye lid, will be tried for better retention without taping rabbit eyes shut, and ocular tissues will be collected for analysis of bacterial quantification in order to better measure the efficacy of formulation.

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