DEVELOPMENT OF A NEW POLYMER OCULAR INSERT TO TREAT FUNGAL INFECTIONS THREATENING THE CORNEA

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Abstract

Fungal keratitis is a kind of dangerous, sight-threatening corneal infections which crucially affects the quality of patients' life, especially in developing countries where it is more prevalent. The infection often has more unfavorable outcomes than other types of eye infections, yet there is not much evidence to present treatment. Currently used methods of treatment applied in ophthalmology are the topical application of a drug in the form of eye drops, liquids, and emulsions. However, the anatomical and physiological structure of the eye restricts the delivery of a therapeutically active drug concentration for ocular disease treatment. Natural barriers protect the eye from different damaging factors while limiting the penetration of a drug.

Consequently, such topical delivery requires increased employment in order to maintain adequate bioavailability and concentration levels. Eventually, it leads to a long-time expensive treatment. Another route is a systemic treatment, which is more effective in comparison with the topical route but limited due to a significant number of side effects.

Thus, it has directed our concerns to develop a new cost-effective drug delivery vehicle, which can provide sustained and prolonged drug release. Over the past decades, the variety of ophthalmological strategies was applied with an attempt to overcome those ocular obstacles and achieve therapeutically most effective transport of the drug to targeted segments of the eye with different drugs used, namely, natamycin, econazole, voriconazole, and ketoconazole.

In this study, using voriconazole as a non-toxic antifungal agent, the new polymer-based drug delivery device was developed, characterized, and in-vitro, in-vivo tested. The system is present in the form of flexible hydrogel rods with a porous structure which are loaded with the drug. This thesis work demonstrates that a polymer ocular insert can significantly reduce the dosing frequency of voriconazole administration and improve long-term patient compliance. The obtained data shows the prolonged release of drugs from the device with a peak concentration of 1.2 mg/mL within the first hour.

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List of Abbreviations and Symbols

DMSO	Dimethyl sulfoxide
EDS	Energy-Dispersive X-ray Spectroscopy
FK	Fungal Keratitis
NVP	1-vinyl-2-pyrrolidinone
TEGDMA	Triethylene glycol dimethacrylate
OCT	Optical Coherence Tomography
PBS	Phosphate buffer saline
PCL	Polycaprolactone
PCR	Polymerase Chain Reaction
PMMA	Poly (methyl methacrylate)
PVA	Poly (vinyl alcohol)
PVP	Poly (vinyl pyrrolidone)
PEG	Polyethylene glycol 1000
SEM	Scanning Electron Microscopy
SNR	Signal-to-Noise
UV	Ultraviolet
VCZ	Voriconazole
XPS	X-Ray Spectroscopy

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Chapter 1 – Introduction

1.1 Fungal Keratitis

Fungal Keratitis infection of the cornea is the leading cause of monocular blindness and eye impairment worldwide. Primarily, it is more often affecting marginalized populations.

The pathogenesis of fungal infection threatening cornea is not well clarified yet. Generally, when the cornea is damaged or experiences trauma (for example, during agricultural work in developing countries) [1], it tends to go under the condition of being infected by fungi or any other microbes leading to fungal keratitis (FK) [2].

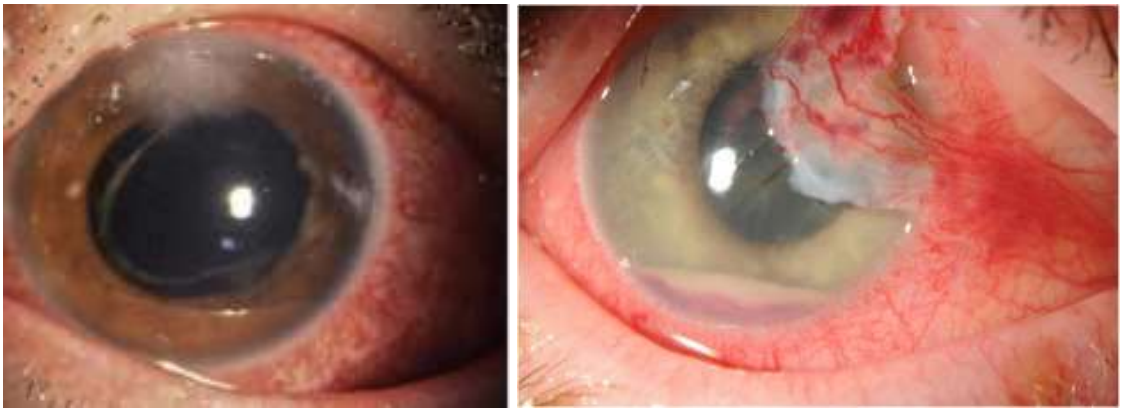
According to the World Health Organization, approximately 285 million people are suffering from various eye diseases, including fungal infections of posterior and anterior segments of the eye. Furthermore, the incidence is estimated to increase substantially in the following 10 years [3]. Studies show that a potential group of people under the risk of corneal infection is people wearing contact lenses, as it could be seen from statistics in the United States, where it is accounted for 37% [1]. In India, the number of cases of FK caused by *Aspergillus* is 113 per 100,000, whereas the United States reports 30,000 cases of infection every year [4]. Timely carried, and precise diagnosis procedure of the problem is vital for FK treatment. Overall, under temperate climate conditions, there is a relatively small number of infection cases, while under tropical climate conditions, it reaches over 40% of infectious ulcers [5]. FK outcomes are worse and less perspective than of bacterial keratitis. The immediate treatment is required in 50—70% of cases, and graft for the cornea is needed in 30 to 54% of cases [6]. Moreover, some studies have revealed that the immune system of a patient plays a significant role in the pathogenesis of the keratitis [7-8]. Nowadays, for the most reliable diagnosis of the FK, there are the following methods used: confocal microscopy, polymerase chain reaction (PCR), and optical coherence tomography (OCT) of the anterior segment. The disadvantages of these techniques are high cost and relatively time-consuming procedures. One further problem that might be faced during the diagnosis of FK is the lack of antifungal drugs in developing countries [4,9]. Conventional ways of diagnosis are primarily based on staining and culturing the material from the cornea. In other words, the diagnosis of FK mainly bases on fungal culture, and this way it is available and easy to confirm the fungal pathogen. However, as this method is conventional and primitive, it poses some disadvantages.

For example, there is a risk of further consequences due to invasive corneal tissue sampling. In addition, it may be time-consuming as well to identify the pathogen, because incubation time depends on the specimen and lasts approximately 1-2 weeks or longer, up to months. [10]

Mainly, threatening factors of infection are hyphomycetes (Aspergillus, Fusarium) and yeasts (Candida albicans and C. parapsilosis). There is also known evidence of Colletotrichum Gloeosporioides, which is a pathogen that caused FK in China [11]. There are two known groups of antifungal agents: azoles (voriconazole, ketoconazole, fluconazole) and polyenes (natomycin) [4].

Overall, the clinical picture of the cornea infection includes sudden pain, reduced vision, especially in sunlight, and inflammation of the cornea surface. Those symptoms are observed in all cases during the studies of patients with FK infection [12].

Specifically, FK includes the following: color change of corneal epithelium with appearing ulcers on the surface, infiltration of the intact epithelial layer, infiltration of the stroma, inflammation, and visually observed fungi hyphae on the cornea [13].



a) b)

Figure 1.1 Cases of fungal keratitits: a) Corneal infection caused by Aspergillus fungi; b) Corneal infection caused by Candida dubliniensis [10, 14]

1.2 Etiology

Ocular diseases from *Fusarium* and *Aspergillus* species are primely associated with agricultural work. *Fusarium* fungi are commonly found in harvest plants, whereas *Aspergill*us fungi can be found in rot plants and soil. Other types of fungi causing FK are *Paecilomyces*

and *Acremonium.* These species show negative response to almost all sterilization methods, even to those that are used during ophthalmic surgeries. The third keratitis-causative fungi in the world are dematiaceous fungi *Curvularia*, *Bipolaris,* and *Exserohilum.* They usually damage the epithelium layer of the cornea. However, in case of delayed treatment, the ulceration may turn into an infection of deeper layers [15]. In developed countries, the prime factor causing FK is yeast, which is caused mainly by *Candida albicans*. Yeasts contribute nearly 30 to 52% of FK observed in geography with temperate climates, namely Europe, the United States and Australia, where it is accounted for 1-5% of all infectious diseases [6]. Infections usually damage the stromal layer, leading to the slow destruction of the epithelium. Furthermore, other types of fungi may cause FK as well, but much rarer in occurrence [16]. Fungal infections require: 1) survival and growth at the cornea, which means that the need to correspond to human corneal temperature is 32.6°C, which is proper for the growth and toxigenicity of two of the most common pathogens of FK; 2) the ability to penetrate in lack of host defenses; 3) the ability to digest and absorb nutrients in the host; 4) the ability to resist host immune system

1.3 Therapeutic routes in ophthalmology

There are three main routes for drug delivery to the eye: topical, intraocular, and systemic routes [17].

Topical administration is the most popular available method of drug delivery to the cornea. Effectiveness of drug application locally (for example, with natamycin 5%) is limited because of poor penetration of the cornea. Voriconazole is a more modern antifungal agent for the treatment of FK, which has better ocular penetration. In addition, it is accepted as the only kind of drug that showed susceptibility to 100% of fungi introduced with keratitis [1]. Topical delivery of voriconazole is usually applied to treat diseases like glaucoma and chronic dry eye that are localized in the anterior segment. It is present in the form of eye drops, and many researchers work on prolongation of drug drops being on the surface of the cornea.

Topically delivered eye drops are quickly washed out into the nasolacrimal channel due to the quick turnover rate and reclamation time of the tear film and are dispensed by conjunctival and lymphatic flowing. Consequently, only 5% is absorbed into the cornea surface tissue, and the anticipated bioavailability of the drug to the anterior segment is less than 5%. For this reason, frequent administration of the drug in the form of eye drops is required. Furthermore, over 18% of patients may acquire microbial contaminants on the eyedrop bottle by touching it with eyes

or face during exploitation. Moreover, for patients, there might be a problem with applying precisely one quantity of drops, which also obstructs the treatment [18]. Summing-up, treatment by topical application of the drug might result in patients' noncompliance.

Intraocular delivery **(**Intravitreal injection) is an effective way to deliver the therapeutic concentration of drug to the posterior segment. Although intravitreal delivery is used throughout the decades, it is the most invasive approach, among others, resulting in potentially severe complications like retinal detachment and cataract [19]. Intraocular delivery is often painful and extremely uncomfortable for patients.

Systemic delivery is effective from the penetration of the drug view, especially in comparison with topical application. However, this kind of treatment emphasizes mostly on the posterior segment. In addition, this way, the drug is not localized or targeted; as it becomes available systematically, a higher dose of the drug is required, which brings a significant amount of adverse side effects [20].

For successful treatment, it is essential to consider the vehicle used for drug delivery. Present pharmaceutical forms of drug delivery involve eyedrops, ointments, hydrogels, insertions, intraocular injections [21]. Eyedrops usage is limited due to low corneal bioavailability, which is <5%. Ointments cause discomfort for patients, particularly eye irritation and blurred vision. Drug delivery, with the help of hydrogels, poses low compliance for patience, as it is still in the process of developing optimal technology. Various inserts used in ophthalmology nowadays are withdrawn due to patients' discomfort and sensitive reaction to the components. As was stated previously, injections and implants are considered an invasive method for ophthalmic treatment.

Hydrogels are widely used in contact with corneal surfaces, namely for correcting the vision by contact lenses. Those hydrogels are modified with better biocompatibility, wearing time, multiple-use and etc. Such characteristics provide a high potential to build drug-delivering devices. While no such device is commercially available for the treatment of the cornea, much research and development work in being done on investigating hydrogels for this purpose [22].

1.4 Physiological barriers to drug delivery

The eye includes three chambers: anterior, posterior, and vitreous (Fig. 1.2). There are also three major layers: fibrous, vascular, and neural. The external epithelium of the cornea has

five sublayers of 50–100 μm thickness. Its lipophilic character confers 90% of the eye's barrier to the penetration of hydrophilic drugs, and the other 10% of it is a boundary for hydrophobic drugs. The second layer is the so-called Bowman's membrane, which is a tiny layer thinner than the first one, and therefore it does not represent a substantial barrier for the penetration of any drug. Thirdly, the hydrophilic layer stroma, which is accounted for 90% of the cornea and is considered as the main barrier for the lipophilic drug penetration, as it contains 80% of water. Further, there is a Descemet's membrane, which does not affect drug delivery. Next, the endothelium that covers the surface of the cornea and is more permeable than the outer epithelium layer [23]. The tear flow rate is 1.2 μL/min. In some cases, it may increase 100-fold due to reflexes of the eye. The local employment of the eye drops or ointments is rapidly flown away by the tear fluid right after application. Besides, over 2 mL of secrete is produced daily by the eye, which forms a hydrophilic layer on the cornea surface and clears pathogens and foreign substances [24]. Moreover, the pH of the tears fluid may also affect penetration. Studies show that the perfect pH for the ocular drug solution should be in the range of 7 and 7.7 [25]. Conveying therapeutic agents with particular intraocular purpose and accomplishing an effective drug concentration is restricted by several natural anatomical and physiological barriers of the eye, including the corneal and anterior segment obstructions and the blood-retinal and the conjunctival barriers.

These barriers form substantial obstacles for drug transport as they constrain the diffusion and penetration of therapeutics. The human eye includes a lachrymal gland, which helps to grease up and clean the cornea from foreign substances by producing tear fluid [26]. These factors restrain drug penetration into or over the cornea resulting in low medicate bioavailability of primarily less than 5% of most locally applied drugs with this esteem even lower for macro size molecules.

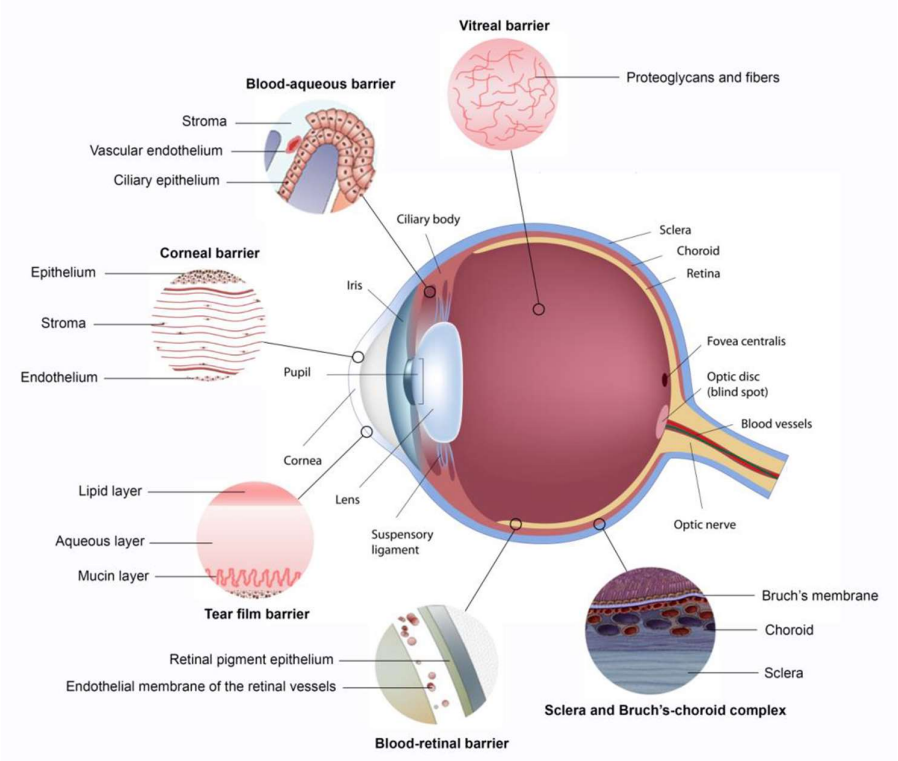


Figure 1.2 Anatomy of the eye as well as drug delivery routes [26]

1.5 Antifungal agents for keratitis treatment

The main objective of the treatment is to conquer the infection and to save the patient's vision. Successful treatment of FK requires antifungal agents as medical therapeutics. The quality of treatment depends on the penetration of the drug to the eye to reach therapeutically effective levels. Nowadays, the scope of antifungal agents is limited, as the penetration of known insufficient. There are three main types of drugs used in medicine against keratitis. Those are polyenes, azoles, and echinocandins.

Amphotericin B was commonly used representative of polyenes group. These drugs have a meager penetration ability, which means that a higher amount of drug is needed in order to reach sufficient concentration levels. In addition, the disadvantage is renal toxicity as a side effect that happens almost 80% of patients. Mainly, the defined concentration of the drug is 0.15%, which is enough to treat through the stroma but ineffective in the case of the intact epithelium of the cornea. Another negative aspect is that Amphotericin B has an antifungal activity to Aspergillus, but no impact on FK caused by Fusarium.

Natamycin is the second representative of polyenes, which is used for treatment in many developed countries. It is suggested to apply a drop per hour maximally. It benefits as it may be used against all main species like Candida, Aspergillus, and Fusarium. However, natamycin has a limited penetration ability, which substantially challenges the treatment.

Another polyene is nystatin, which is not used frequently in medicine because there are other, more potent agents for this purpose.

Among azoles groups, there are ketoconazole, miconazole, fluconazole, and voriconazole. Econazole and ketoconazole have a full spectrum of activity against all prime fungal infections caused by Aspergillus, Fusarium. However, a high amount of these preparation results in severe unfavorable consequences like impotence and gynecomastia. Econazole is known as causing eye irritation.

Fluconazole is effective in topically administered therapy as well as systemic application. It benefits with its safety, non-toxicity, yet it has inactivity against main species of keratitis infection, such as Aspergillus and Fusarium. Besides, it has limited activity against Candida glabrata and Candida krusei [13].

Voriconazole is a modern triazole antifungal agent with a broad spectrum which is used in the treatment of FK infection caused by Candida, Fusarium, and Aspergillus species. Voriconazole acts by inhibiting the 14-alpha-lanosterol demethylase enzyme in the process of fungal cell development. Nowadays, voriconazole is available as an oral drug form as well as the solution for injections into veins (systemic administration).

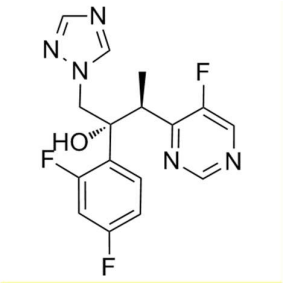


Figure 1.3 Molecular structure of voriconazole

However, such delivery methods of the drug are causing several side effects. For example, vomiting, diarrhea, headache, loss of appetite, and tiredness. Beside unfavorable consequences, the drug is insoluble in water, and it tends to the first-pass metabolism. Therefore, voriconazole administration in the form of topical delivery has become popular

among clinicians. As for all locally applied drugs, voriconazole penetration to the eye is limited. Permeation enhancers might be used to improve permeability; however, they cause skin and eye irritation as a side effect. Thus, local drug delivery devices are found to be an optimal method of increasing permeability level and providing prolonged release of the drug topically. [27]

Currently used methods of treatment with voriconazole drugs against FK are expensive and time-consuming. Voriconazole, a triazole antifungal agent, gained considerable attention as a treatment of human and equine FK because of its high corneal penetration and 100% antifungal activity against the known primary pathogens. [28]

1.6 Polymers used in ophthalmology

There is a variety of biomaterials, especially polymer-based materials for ophthalmological purposes. These materials must comply with all the requirements for the ophthalmological application. In accordance with polymer classification, biopolymers might be divided into natural, synthetic, and modified polymers. Some of them are biodegradable, but most of the polymer ocular biomaterials in clinical use are stable, so non-biodegradable. While choosing the kind of polymer for drug delivery, the administering site must be considered first. Therefore, for some targeted drug delivery polymers must be mucoadhesive, to interfere with the cornea. In another case, it is essential to have a controlling ability to release. Another significant aspect of the requirements is biocompatibility. Tolerability, irritation of the eye surface, and cell toxicity must be considered for each polymer in particular. Examples of ophthalmologically approved polymeric systems are the following: alginate, chitosan, PEG, gelatin, ethylcellulose, albumin, PCL, PLA, PMA, and other formulations [21].

The sustained and prolonged release extends the time of systemic drug levels within the therapeutic range and reduces the number of doses the patient must receive to maintain a therapeutic effect, thus increasing patients' compliance [29].

Designing the ideal ocular device or drug delivery system requires knowledge, not only the therapeutic mode of action but also the composition that makes the vehicle. Hydrogels can be divided into made of natural or synthetic monomers. Examples of natural polymers are hyaluronic acid, chitosan, collagen. Synthetic polymers are those that are not found in nature;

for instance, those are poly (ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), and poly(methyl methacrylate) (PMMA) [30].

Pointing to overcome the significant therapeutic barriers, there was extensive work done in the investigation within the field of ophthalmic drug delivery during the last two decades. From this point of view, polymers are considered to be a crucial aspect due to its potential designing ability, flexibility, and modularity.

1.7 Justification of the study

Taking all of the above-stated difficulties of FK treatment into account and considering that currently, there is no successful drug - delivery device commercially available for the treatment of the corneal fungal infection, the objective of this thesis work is to develop a new cost-effective drug delivery vehicle, which is anticipated to have sustained drug release, high drug penetration and better bioavailability.

For this purpose, following methodology is suggested: (1) preparation of solutions, (2) design and synthesis of rods, (3) characterization of rods, (4) in-vitro study, (5) microbiological experiment, and (6) in-vivo study.

Chapter 2 - Materials and methods

2.1. Materials

Voriconazole as an antifungal agent was provided by Hyper Chemicals (Hangzhou, China), 1-Vinyl-2-pyrrolidinone as a monomer for synthesis (>99%), triethylene glycol dimethacrylate (99%) as a cross-linking agent, tert-butyl peroxybenzoate (98%) as an initiator, dimethyl sulphoxide (>99.9%) as a dissolver for the drug and phosphate buffer saline tablets were obtained from Sigma Aldrich (Saint Louis, Missouri, USA), polyethylene glycol 1000 as a pore generator was purchased from Millipore Corporation (Berlin, Germany)

2.2. Preparation of solutions

Voriconazole (VCZ) solution was prepared by dissolving 1 g of voriconazole to 5 g of dimethyl sulphoxide and stirring for 15 min on a magnetic stirrer at room temperature. DMSO was chosen as a solvent for voriconazole based on literature. Then, for further experimental analysis, the solution was diluted when needed. For the 6 g of monomer solution, 3,45 g of 1-Vinyl-2-pyrrolidinone (NVP) was mixed with 1,5 g of triethylene glycol dimethacrylate (TEGDMA) and 1.05 g of tert-butyl peroxybenzoate. To make the material 10% porous, 0.6 g of polyethylene glycol (PEG) was added to the solution. The solution was left under room temperature until the complete dissolving of PEG. Phosphate buffer saline (PBS) was prepared as predetermined in instructions. One tablet was dissolved in 200mL of deionized water at 25^{o}C.

2.3 Synthesis of polymer rods

Polymer rods were synthesized in special capillary tubes 75 mm long with the inner diameter of 1,1-1,2 mm and the outer diameter of 1,5-1,6 mm. The tubes were sealed from one side by the Bunsen burner (Fig. 2.1). Then the tubes were filled with a monomer solution and put in a vertical position in a water bath set at 80^{o}C for 40 min on a magnetic stirrer.

Afterward, the capillary tubes were gently broken, and hydrogel rods were taken out. Synthesized rods were washed out with distilled water multiple times to free the pores and left to dry on soft tissue paper. Next, the dry rods were put into the voriconazole drug solution (5:1) and left for 24 hours to load the drug according to the 'breathing in' technique. Following this, drug-loaded rods were additionally put into distilled water. The drug was seen to crystallize immediately upon contact with water. The rods were kept immersed in water for 24 hours to wash out DMSO. The water was removed by fresh distilled water every 6 hours. The prepared rods are transparent, flexible, and soft. After contact with water, the color turns into white, and the material becomes swollen; while it dries, it becomes strong and stiff and turns back to transparency. To define the optimal flexibility and stiffness, by the trial-error method, the synthesis was carried multiple times with different synthesis conditions, namely time and temperature. The optimal structure and flexibility were defined visually and practically. (See below).

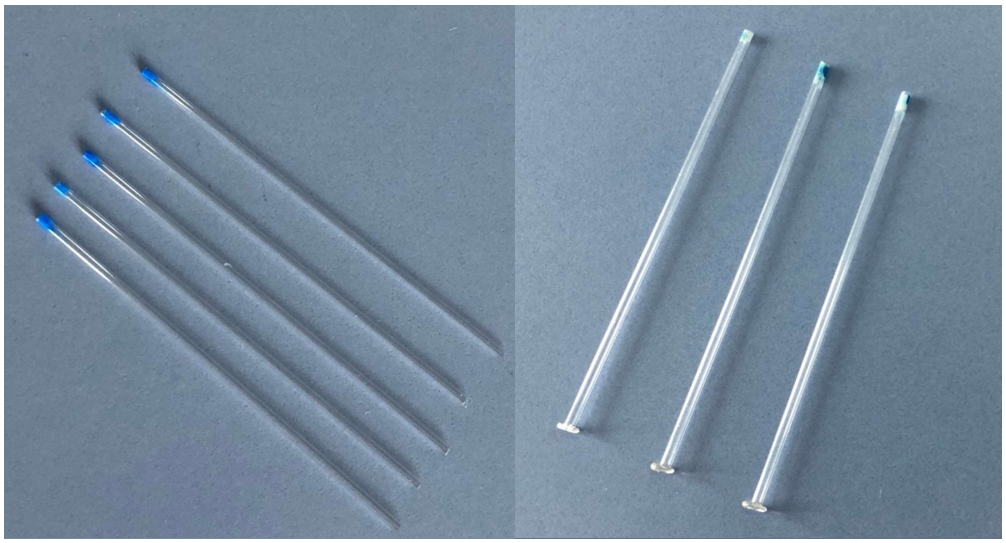


Figure 2.1 The capillary tubes used for the synthesis of polymer rods. Rods are closed from one side (right)

2.4 Characterization of the prepared polymer rods

For the characterization of synthesized polymer rods following techniques were used: Scanning electron microscopy (SEM), Energy dispersive spectroscopy (EDS), X-Ray

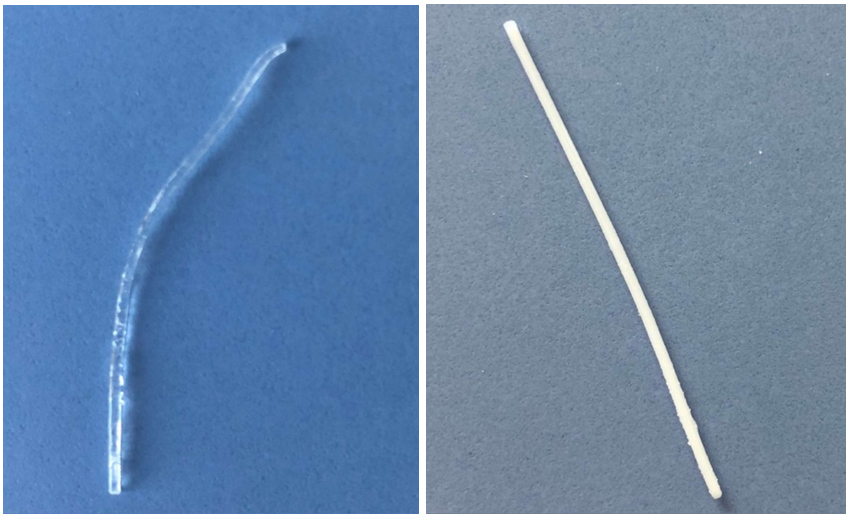
photoelectron spectroscopy (XPS). Synthesized polymer rods were analyzed under UV light source in order to verify the presence of voriconazole drug through the comparison of drug-loaded and drug-free samples.

2.4.1 Scanning electron microscopy characterization (SEM)

Surface morphology and porosity of the samples were examined by using SEM (Crossbeam 540, Zeiss Gemini 2, Germany). Images with different magnifications were obtained. Prior to examination by SEM, each specimen was sputter-coated using carbon for 120 s at 1 kV and 20 mA by Turbo-pumped sputter coater (Quotrum Q150T ES, UK). Such coating of the polymer rods is required to prevent charging of the sample and to increase the number of secondary electrons, and it increases the signal to noise ratio (SNR).

2.4.2 Energy dispersive spectroscopy (EDS)

The EDS was applied for semi-quantitative analysis to identify particles on the surface and inside the cross-section of the rods. It is known that the voriconazole solution contains fluorine groups, which will help to identify the existence of drug particles inside the pores of the device. The specimens were coated with carbon beforehand by Turbo-pumped sputter coater.



a) b)

Figure 2.2 Synthesized drug-loaded polymer rods shown in different states: a) Dry form; b) Wet form;

2.4.3 X-Ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron experiments were conducted in the Wenzhou Medical University by using a Thermo spectrometer (Al K α X-ray source). Drug-free and drug-loaded samples were studied at the angle of 90°. Elemental composition was determined with binding energy in the range of 0 and 1200 eV. XPS analysis was carried in order to confirm the composition on the surface of synthesized polymer rods with and without the drug.

2.5 Drug release studies

Drug release was studied through in-vitro and microbiological experiments. DMSO was chosen as a solvent for the analytical method. Phosphate buffer saline (pH 7.4) was used as a tear simulating media for drug release and permeation studies. A standard 1000 μg/mL solution of drug in DMSO was prepared for estimation of λ_{max}. Solutions of concentrations from 10–200 μg/mL were prepared by diluting in PBS, and the absorbance was measured from 200 to 400 nm in UV-VIS spectrophotometer (photoLab 7600 UV-VIS). The calibration curve was plotted for these concentrations in order to obtain a linear equation and the correlation coefficient. All experiments were conducted multiple times.

2.6.1 In vitro study of drug release

Two approaches were applied to calculate the concentration of VCZ in polymer rods. For the first drug release analysis, several PBS solutions were prepared as media mimicking the tear fluid. Drug-loaded samples were put into each of 10mL PBS solution bottles. Then, they were stored in the thermostat with set temperature 36 ± 1 °C during the following periods: 15 min, 30 min, 1h, 1,5h, 2h, 3h, 4, 12h, and 24, and then the samples were scanned (Fig.2.3). The measurements in the range of 200-400 nm of the absorbance were taken on the UV spectrophotometer (photoLab 7600 UV-VIS) at the predetermined time interval points.

The second method for drug release study of polymer rods was carried out by using PBS solution, as it was used as the medium for drug release study at the temperature corresponding to eye temperature. Drug-loaded samples were put into 10 mL of PBS and stored in the thermostat with set temperature 36 ±1°C, and then PBS solution was replaced by a fresh portion. Measurements were taken at a predetermined time interval of 6 to 48h. In the first

twenty-four hours, the release was measured every 6 hours, then once a day. A UV spectrophotometer measured the absorbance of the drug in the range of 200-400 nm.

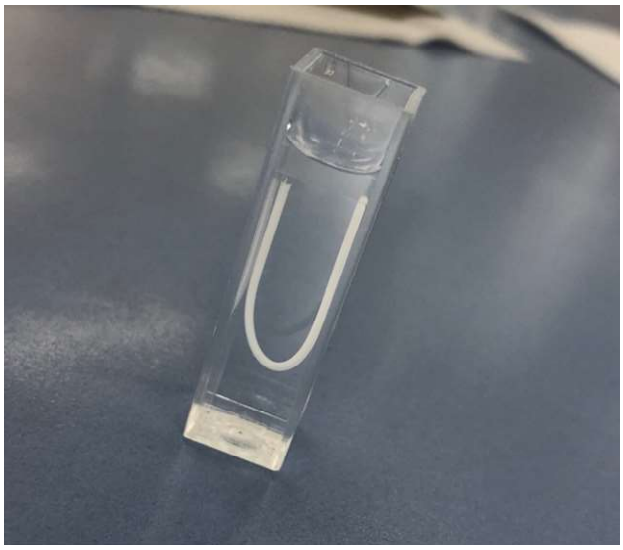


Figure 2.3 Drug-loaded polymer rod in the cuvette for continuous release study

2.6.2 Microbiological study of drug release

Aspergillus and Fusarium from patient origins were obtained from the Eye Hospital of Wenzhou Medical University, China. The antifungal activity was carried out by the diffusion method. The fungal strains were on storage at 40°C, which is an optimal condition for the fungi species. Polymer rods were placed on the Petri dish, where the fungi were spread. Drug-loaded rods were used as a positive control, and drug-free rods were observed as a negative control. All plates were maintained at 37 °C and stored for the one-week period of incubation. These microorganisms were put to media, and after incubation, both fungi demonstrate noticeable inhibition of growth.

2.7 In-vivo study with rabbit as a model

A preliminary in-vivo study was carried out in the Eye Hospital of Wenzhou Medical University, China, by professional ophthalmologists. A rabbit with no signs of ocular infection

was used for the study. Before the in vivo experiment, the rabbit was sedated and placed on the table. Then, the ocular polymer rods were inserted directly into the rabbit cornea of one of the eyes. Next, ophthalmologists fixed the device by a particular medical suture thread and examined the handleability of the insert for future applications. In addition, doctors evaluated in a very preliminary experimental setting, the possible risks of eye irritation by the device.

Chapter 3 - Results and discussion

3.1 Synthesis of polymer rods

After the trial-error method of defining optimal time and temperature to obtain proper mechanical properties and structure, the following conditions were chosen: optimal temperature 80°C and synthesis time 40 minutes. Desired properties were defined quantitively by visual assessment. The rods initially are transparent, but after the contact with water, it becomes white (Fig.3.1). This can be explained because of the exchange of PEG molecules with water after washing of polymer rods.

Optimized ocular inserts prepared in this work confirmed required mechanical properties and forming abilities of NVP and proved its potential application in ocular drug delivery. The first visual examination demonstrated the ability of polymer biomaterial to produce strongly structured rods that were adequately flexible and easy to handle.

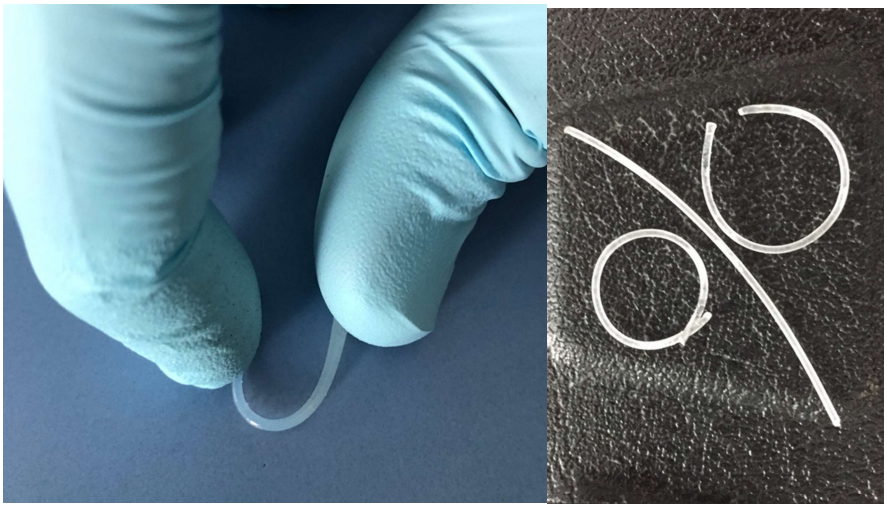


Figure 3.1 Bended wet and circled dry polymer rods performing excellent mechanical properties

3.2 Characterization of synthesized polymer rods

First, by using UV lamp, polymer rods were examined for the determination of drug presence. VCZ provides fluorescence due to a double bond in its structure. It absorbs UV light at 256 ± 1 nm and emits light approximately at 370 nm, which is in the range of visible blue light. The difference is shown below in Figure 3.2.

Figure 3.2 Polymer rods examined with UV lamp: drug-loaded (top) and drug-free samples (below)

3.2.1 Scanning electron microscopy (SEM)

The SEM images of all the polymer rods revealed general smoothness of the surface as well as the open porous structure inside the cross-section of the samples, as it is demonstrated in Fig. 3.3. For this purpose, the rods were partially cut and partially broken, as it is shown (Fig. 3.7). Due to the eye sensitivity, it is necessary that the ocular inserts aimed for topical drug administration are smooth enough and causes no irritation of the eye surface for the patient. The porous structure is required for further drug loading and consequent release. Approximate pore size distribution is in the range of 600-2500 nm. Images are taken at different magnification. Two different groups of synthesized samples were monitored to see the

difference in structure: dry form (before water contact), which contains PEG particles inside the pores; and wet form (after water contact), which is supposed to have open structure due to washed PEG. The difference is shown on Fig 3.4.

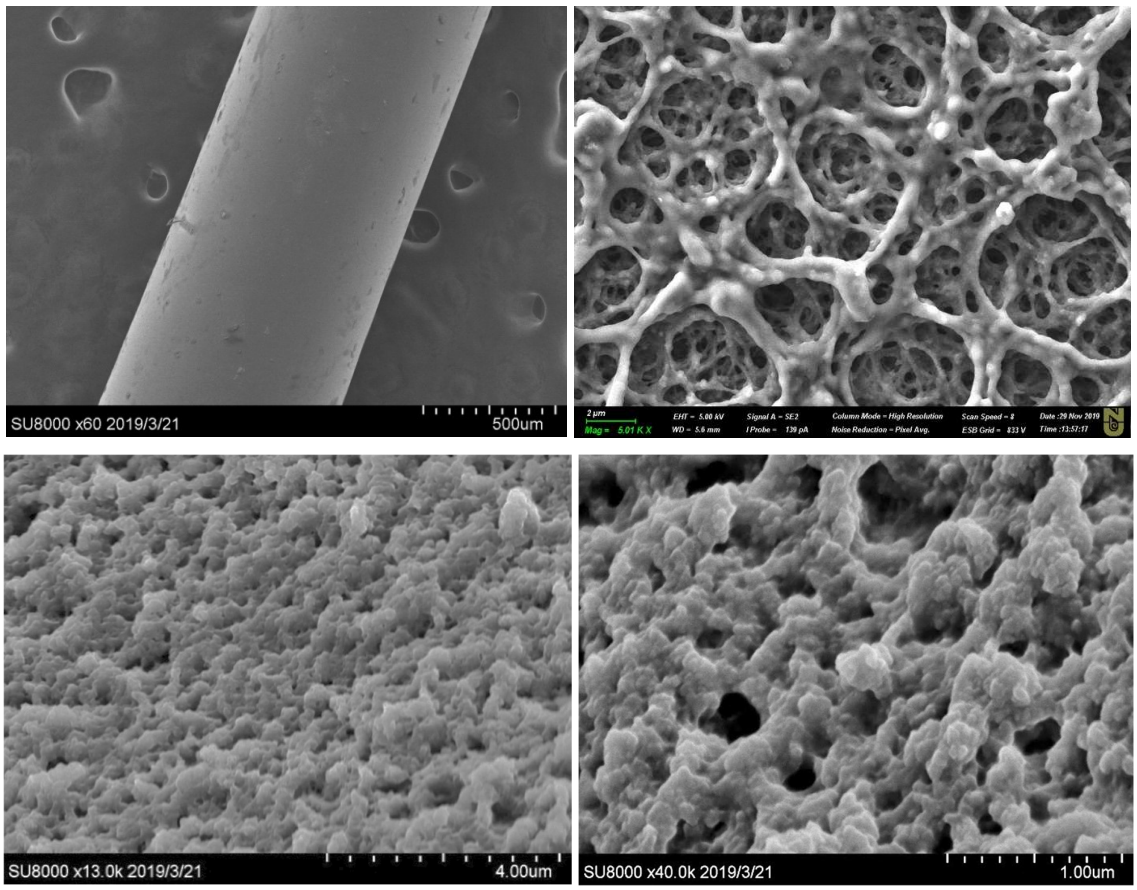
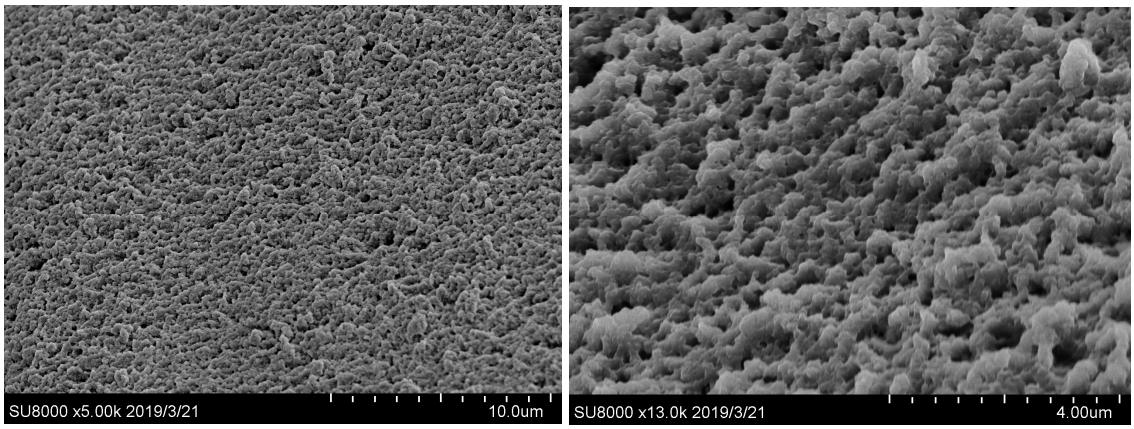
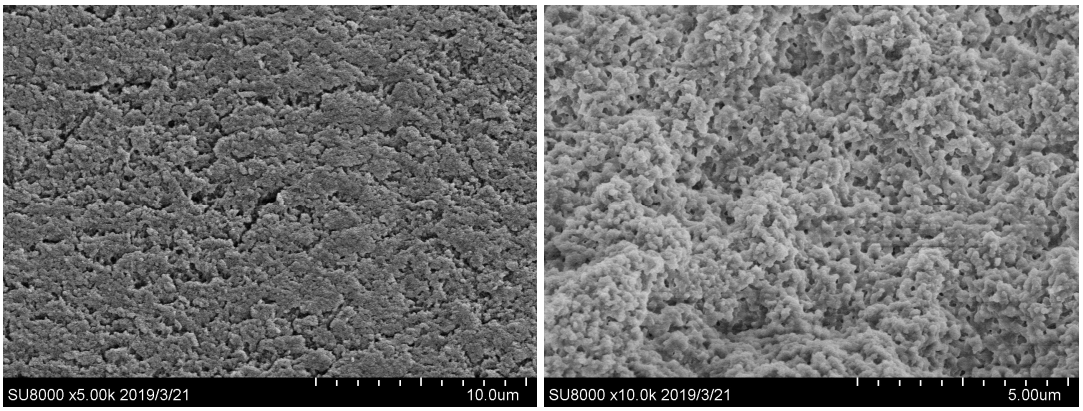


Figure 3.3 SEM images of the surface and porous structure of the polymer rods



a) images of washed with water polymer rods



b) images of dry polymer rods (no contact with water)

Figure 3.4 SEM images of two different group of rods

3.2.1 Energy dispersive spectroscopy (EDS)

EDS is capable of identifying major and minor elements of concentrations higher and less than 10 wt%, respectively; thus, concentrations below 0.01 wt% cannot be identified. This method produces a map of elemental distribution, where dots represent each particular element in different colors [31].

After chemical analysis of the particles in the cross-sectional area by semi-quantitative EDS analysis at 8 kV acceleration voltage following results were obtained. Primarily, it occurs to have expected polymer identified elements, namely high carbon (70 ± 5 wt%) and oxygen content 21 ± 3 wt%) in their molecular structure. In addition, there was 2.5 ± 1.5 wt% of fluorine, which proves the presence of the drug inside the pores (Fig. 3.6) as it is known that voriconazole contains three fluorine atoms.

There is a noticeable presence of the drug; the voriconazole crystals are bright-red (Fig 3.7). A negligible amount of sulfur (0.1% wt) was detected by EDS, which is due to the existence of unwashed particles of DMSO dissolver for the drug. The high presence of carbon is determined by the sputter coating using carbon.

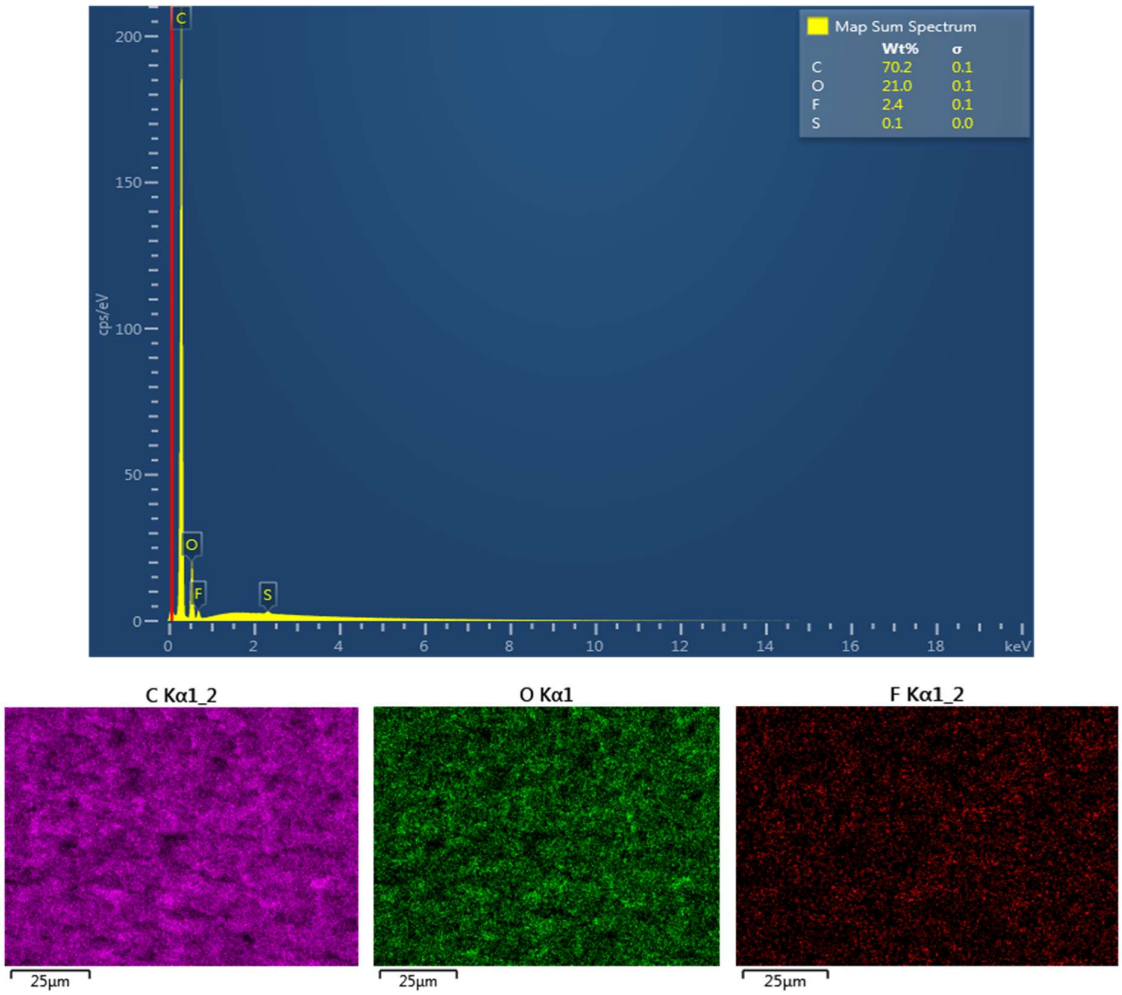


Figure 3.5 Representation of map sum spectrum of drug-loaded rods gained during element analysis by EDS

In comparison, the map spectrum of the empty samples was obtained as well. As can be seen from Fig. 3.6, the absence of fluorine proves the composition of drug-free samples.

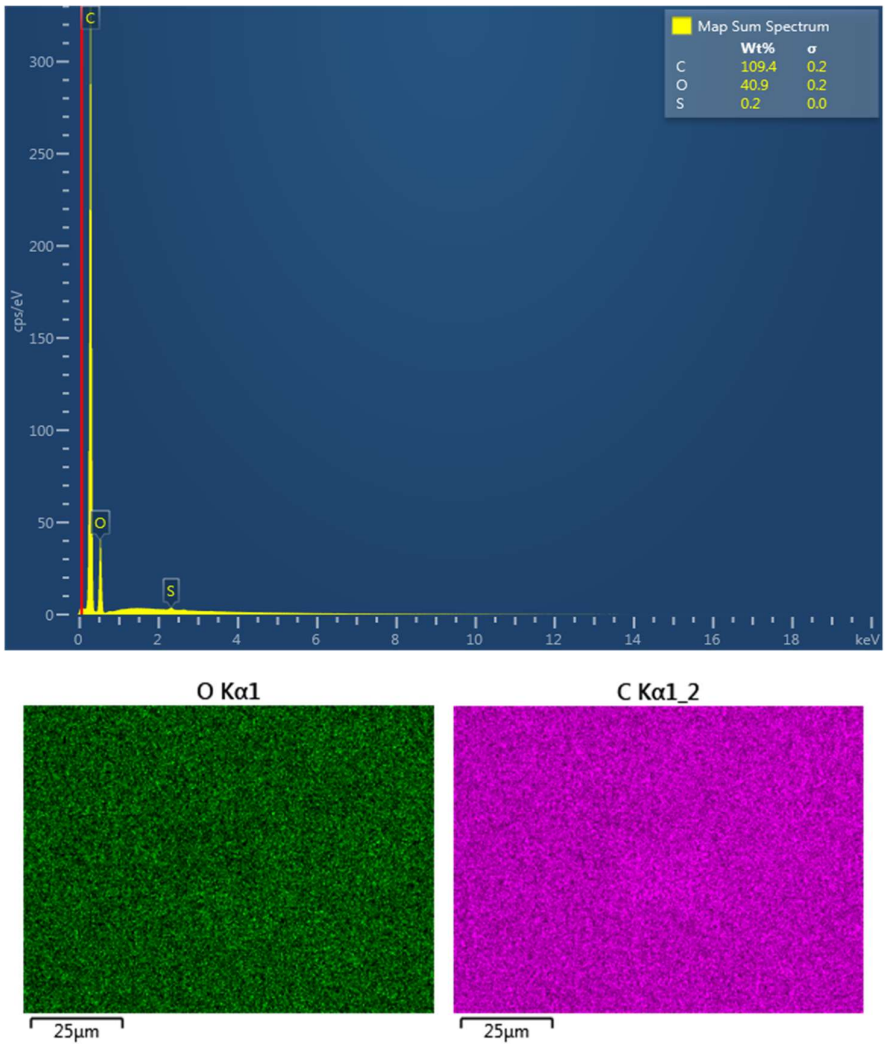
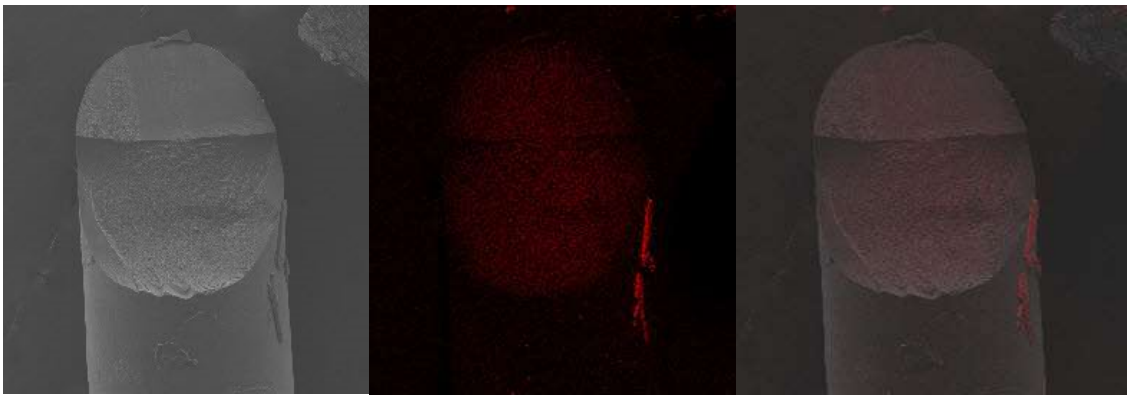


Figure 3.6 EDS map spectrum of drug-free samples



a) b) c)

Figure 3.7 SEM-EDS images of polymer rods: a) Cross-sectional area of the sample; b) Fluorine detected in the pores; c) Superimposed image of the rod with an element distribution map.

3.3 X-Ray Photoelectron Spectroscopy (XPS)

In order to analyze the chemical composition of synthesized polymer rods, XPS experiments were carried out. As seen in Fig. 3.8, in a survey scan of samples C1s (284.0 eV), O1s (531.0 eV), and N1s (398.0 eV) peaks were observed.

The prime components for each specimen are C-C and C-H (284.0 eV), C=O (531.0 eV) and C-N (398.0 eV), which constitutes its intended composition of polyvinyl pyrrolidone (PVP).

In comparison, chemical analysis of drug-loaded samples was conducted as well. As can be seen from Fig. 3.9, one more peak is observed at 686 eV, which is responsible for F1s. That means that some quantity of drug is present on the surface of samples, as VCZ contains C-F in its content.

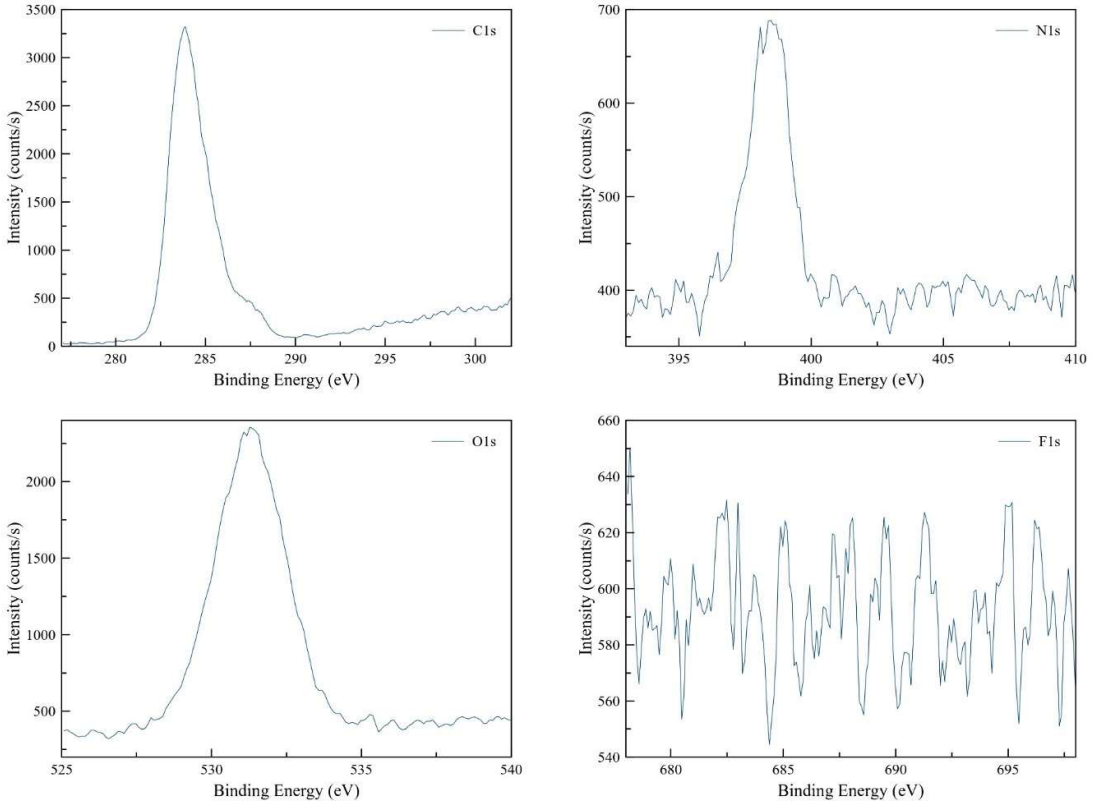


Figure 3.8 XPS analysis of the chemical composition of drug-free samples

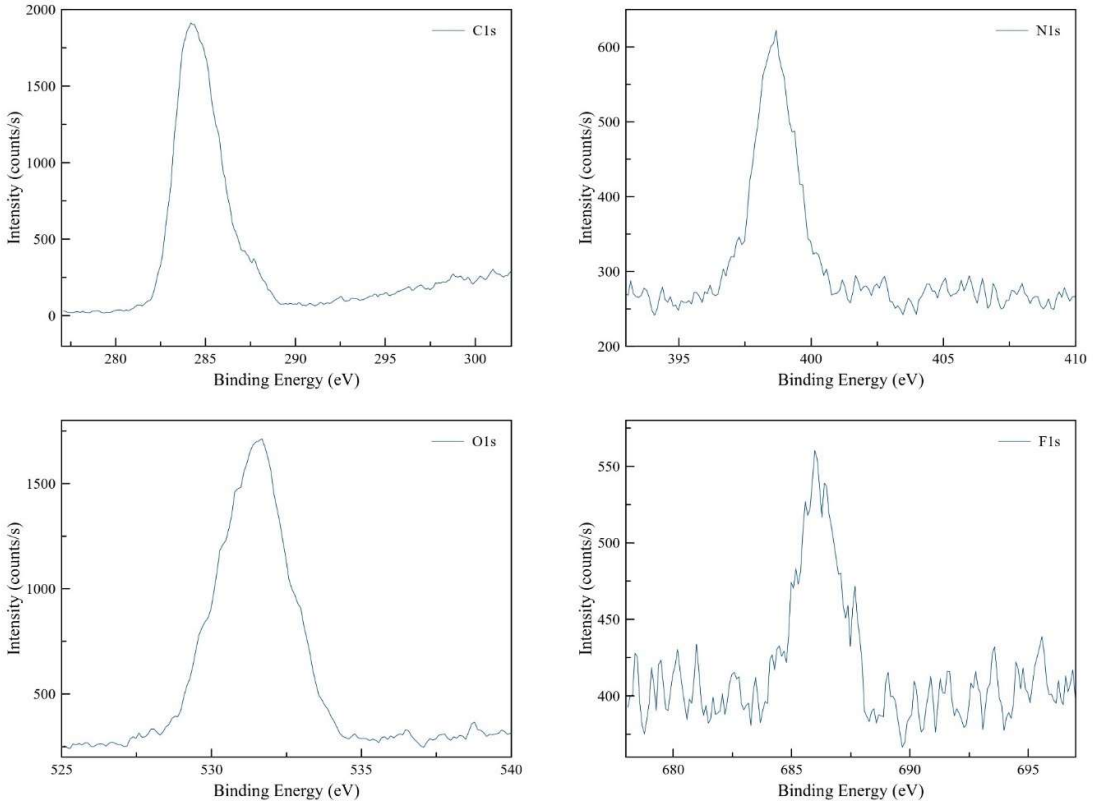


Figure 3.9 XPS analysis of the chemical composition of drug-loaded samples

3.4 Drug release studies

3.4.1 In-vitro study the drug release

In-vitro release experiment provides data about the drug release from the polymer formulation of the prepared device. A calibration curve was produced using standard solutions of voriconazole through a concentration range of 10-100 µg/mL, and the mean was $R^2 = 0.9964$. Concentration range is chosen based on monitoring UV extinction of VCZ; higher than 100 µg/mL concentration showed no change in absorbance and higher wavelengths. The absorbance of prepared solutions was observed at λ_{max} (256 nm), and calibration curves were obtained by plotting corresponding absorbance vs. concentration on x- and y-axis, respectively

(Fig. 3.11). Then, the concentration was calculated by substituting absorbance value to the equation gained from Fig. 3.10.

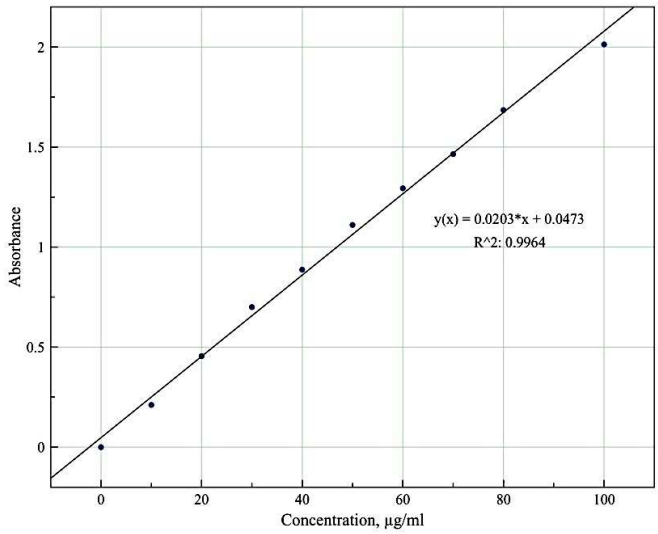


Figure 3.10: a) Calibration curve of VCZ drug

Dependence between concentration (mg/mL) and time (h) was plotted, as shown in Fig. 3.11. The zero-order spectrum was obtained with λ_{max} at about 256 ± 1 nm within all prepared solutions for the first method. It can be seen that the highest point of concentration was reached after 5-hours.

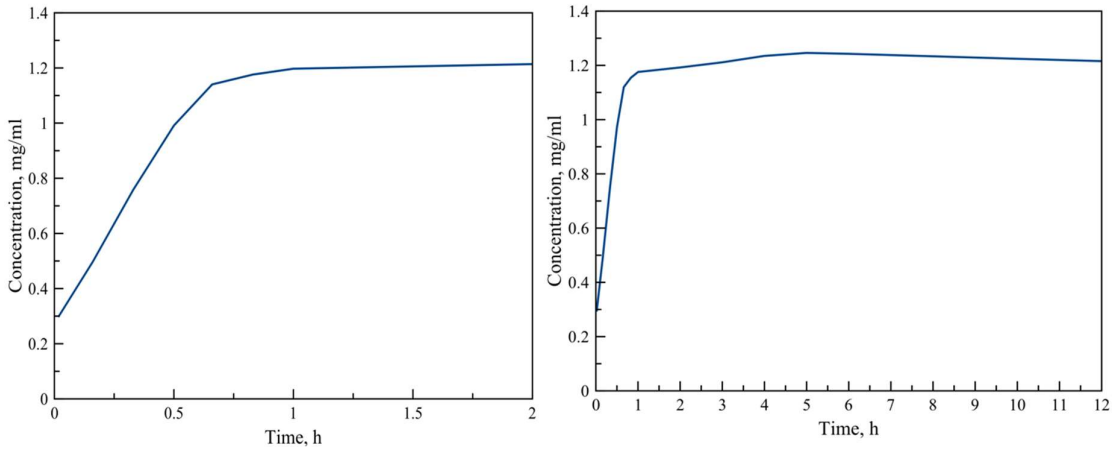
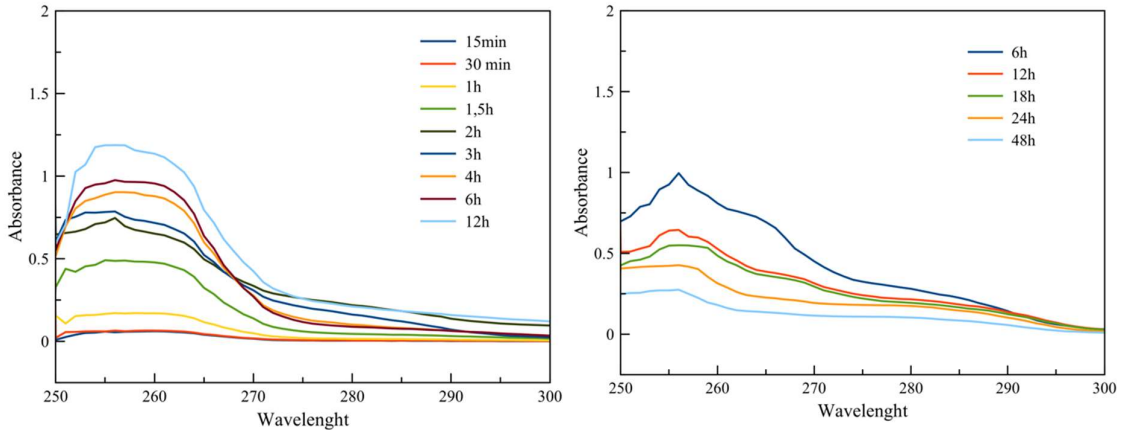


Figure 3.11 Concentration – Time profile for VCZ

The data for the first approach of drug release, where VCZ released continuously from polymer rods during 12 hours at a set temperature of 37°C, shows that with time absorption of the drug increases, which proves the diffusion of the drug in PBS (Fig. 3.12 (a)).



a) b)

Figure 3.12 UV spectra for drug release of VCZ: a) cumulative method; b) refreshment method

The second approach of drug release study, where PBS solution for a drug-loaded polymer rod was refreshed and measured, shows that after 2 days, there was a limited release of the drug (Fig. 3.12 (b)). These studies can be limited due to the solubility of the drug in PBS, as the volume of the latter used in the experiment is higher in comparison with the tear fluid of the human eye.

The volume of PBS is 3 mL, and the length of a rod is approximately 20 mm. The amount of voriconazole released from 1 polymer rod during the first hour is 1.175 mg * 3 = 3.525 mg (from Fig 3.12). In comparison with current eye drops, 1 eye drop with a volume of 0.05 mL, containing 0.5 mg of VCZ drug, that can be delivered to ocular surface ideally (if ignoring the loss due to several barriers). So, 3.525 mg of the drug can be released from the device and 0.5 mg from an eye drop. The ratio (polymer rod : eye drop) would be 7 : 1. The difference between the administration of voriconazole via eye drops and the polymer rod are summarized in Table 1:

Table 1 Comparison of voriconazole in the form of eye drops and polymer rods

Administration of voriconazole	**Via eye drop**	**Via rod (length 20 mm)**
Dose	0.5 mg	3.5 mg
Release time	Immediately	60 min (zero-order)
Bio-availability	Limited due to lacrimal wash-out	Constant during 60 min; diffusion-controlled release and lacrimal wash-out occurring simultaneously

It is anticipated that the release of the drug from the porous rod brings advantages regarding the bio-availability. During the first 60 min after insertion of the rod, the drug is expected to be released continuously (in parallel with the in vitro data). Several aspects must be investigated in much more detail. For instance: (i), are the drug release kinetics comparable in vitro and in vivo? (ii), do elevated concentrations of voriconazole occur? And –if so- can they damage the epithelium (through cytotoxicity for epithelial cells)? (iii), does the presence of the rod impede the lacrimal tear flow? If so, how?

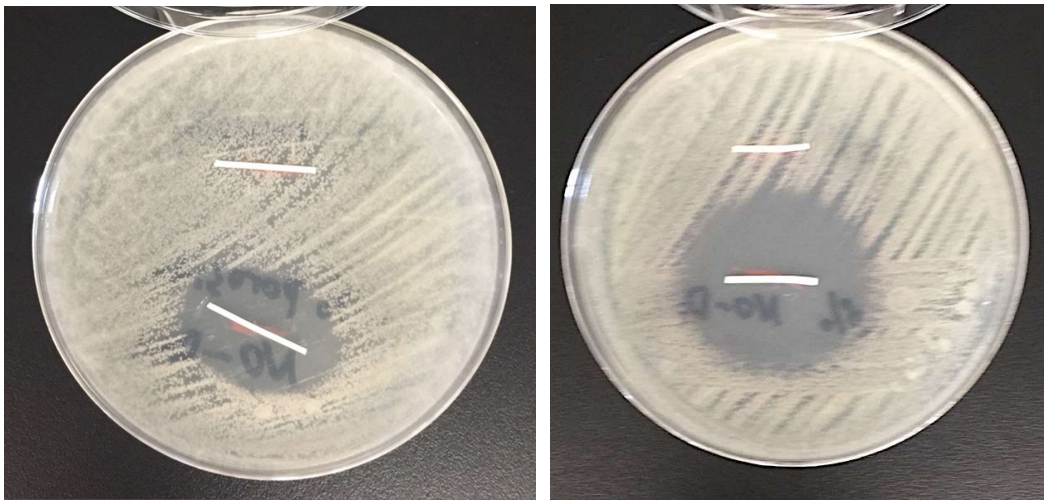
As was stated before, drug delivery through the topical application of eye drops is limited due to low bioavailability. In the case of the drug delivery device in the form of polymer rods, the loss of drug might still be present. However, because of the targeted location of the device, the drug diffuses directly to the infected site of the cornea. Thus, such local administration of the drug benefits in bioavailability and leads to higher therapeutic efficacy.

3.3.2 Microbiological study of drug release

For microbiological testing, following filamentous fungi, Aspergillus and Fusarium were used because they are known as the most common factors causing ocular FK. After the incubation period, the rods were put on the plates for another twenty-four hours, and the zones of inhibition around the rods were measured visually and photographed. Thus, the zones of inhibition prove that the fungi were killed due to the diffusion of VCZ drug from the drug-loaded sample. The zone of inhibition is in the shape of an ellipse in accordance with the shape of the rod. There is a remarkable difference in the area of inhibition zone between 10% porosity

and 20% porosity polymer rods for both Aspergillus and Fusarium species (Fig. 9). It proves that 20% of porosity drug-loaded rods have a much higher amount of drug-loaded and released, as the zone of inhibition around the rod is broader than with 10% porosity. Besides, it was revealed that there is a dependence between time and inhibition zone, as the latter broadens with time.

a)



b)

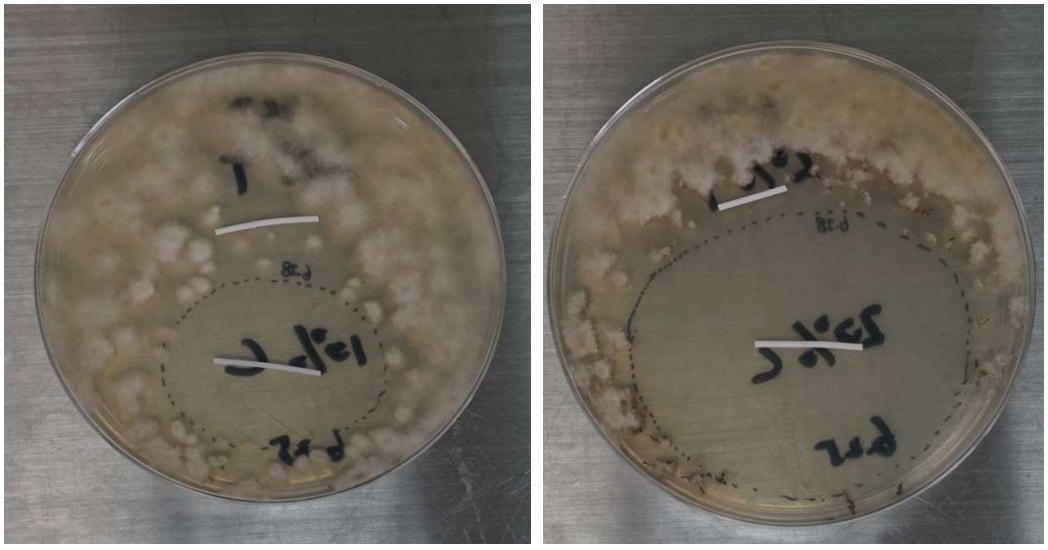


Fig. 3.13 The inhibition of fungal growth around drug-loaded rods a) 10% and 20% porous rods against Fusarium; b) 10 and 20% porous rods against Aspergillus.

3.4 In-vivo study with rabbit as a model

A very preliminary experiment was performed regarding the assessment of the possible utility of the porous rods in clinical practice. In other words, to answer the question: can this approach work in the clinical practice of ophthalmology? Fully hydrated rods, which were 10% porous and uncharged (no drug present) were used in these experiments. An experienced ophthalmic surgeon inserted several of our rods in several rabbit eyes. The surface caused no eye irritability. The structural flexibility of the rod was much appreciated; this feature allows bending of the rods in any desired curvature. It was seen that the rod keeps a tendency to straighten back to its original geometry. This could be prevented by fixing the rod in place, using a microscopic stitching technique (which is routine in ophthalmology). See Figure 3.14. It was concluded that such fixing is not acceptable from a clinical point of view. If the device is to be placed on the cornea for 60 min, the stitching would be an unfavorable option. Moreover, it was concluded that the rod in its present form is too thick (ca. 1.5 mm in the hydrated form). Obviously, all these observations require further optimization of our concept. In particular, the use of circular capillary and thinner glass tubes (rather than straight ones (vide supra)) must be explored.

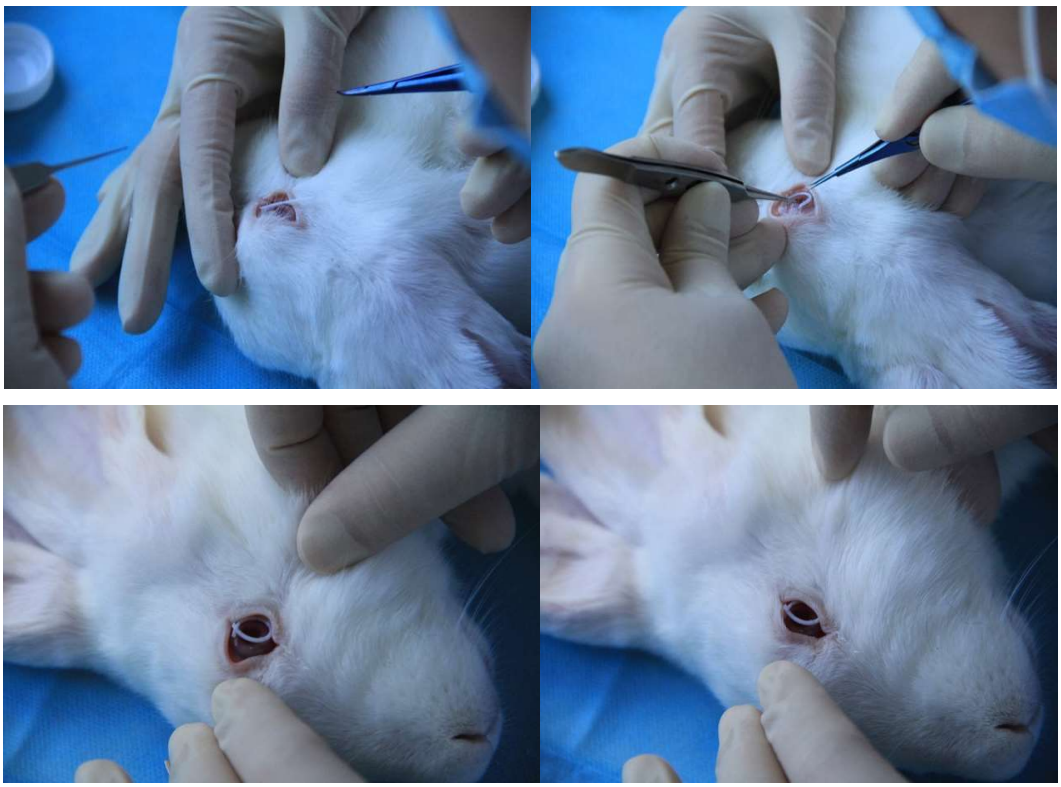


Figure 3.14 Ophthalmological procedure of inserting the polymer rod into the rabbit's eye

Conclusion

This research work demonstrates that the concept of using porous hydrogel polymer rods as a vehicle for controlled topical drug release is technically feasible. The concept was shown with the drug voriconazole, which is an antifungal agent that finds use in the clinic, in the treatment of fungal keratitis.

It is possible to load the rods with an amount of crystalline voriconazole that is clinically relevant. Release studies in vitro show that zero-order drug release can be achieved. A drug concentration of 1.175 and a peak concentration of 1.250 mg per mL is achieved after 1 and 5 hours, respectively. To compare with currently used eye drops of the same voriconazole drug, the ratio of concentrations is 7:1 (device : eye drop). Thus, a polymer rod can be considered as an effective drug delivery device.

Performed SEM, SEM/EDS, and XPS analysis showed the composition of synthesized polymer rods, which corresponds with the initial purpose. Microbiological experiments with fungus revealed successful drug release and the possibility of the material to load drugs. The material consists of PVP, and it is known as commonly used biomaterial in ophthalmology, which is unlikely to cause unfavorable consequences. Furthermore, it was proved by the in-vivo study as well. It revealed the feasibility of the device with no significant adverse eye reaction and thus, confirmed its future potential use in ophthalmology.

Technically, further optimization of our concept is needed. In particular, the use of circular capillary and thinner glass tubes (rather than straight ones (vide supra)) must be explored. In addition, it must be investigated in what sense the presence of a rod on the corneal surface disturbs the flow of the tear fluid. Biologically, it must be investigated whether or not high local concentrations of voriconazole can occur on the corneal surface, and –if so- whether this poses any risk to epithelial cells. This is the most important safety issue.

Ultimately, there is much research work in the development of the most cost-effective and convenient ocular device is required, as the concern of treatment of fungal keratitis is relevant all over the world. Proposed in this study, polymer rods as ocular inserts have the potential to

solve this problem, although further work on possible complications of the device must be considered in future studies.

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