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In Vitro and *In Vivo* Biological Assessment of Dual Drug-Loaded Coaxial Nanofibers for the Treatment of Corneal Abrasion

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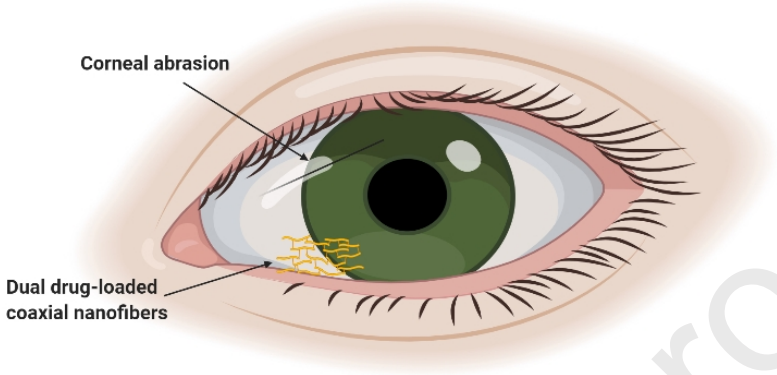
Abstract

The treatment of corneal abrasion currently involves the topical administration of antibiotics, with moxifloxacin HCl (0.5% w/v) eye drops being one of the most widely used treatments. Our previous work (Tawfik *et al*., 2020) involved the development of coaxial poly-lactic-co-glycolic acid (PLGA) and polyvinylpyrrolidone (PVP) nanofibers loaded with the antibiotic moxifloxacin HCl and the anti-scarring agent pirfenidone in the core (PVP) and shell (PLGA) respectively, with a view to the system comprising an ocular insert for the combination therapy of corneal abrasion. In this study, we examine the antimicrobial, anti-scarring and pharmacokinetic properties of the fibers alongside consideration of their toxicity and propensity for irritation. Minimum inhibitory concentration and zone of inhibition studies against *S. aureus* and *P. aeruginosa* were performed, while fibroblast cell viability and α-smooth muscle actin (α-SMA, a biomarker for scar formation) were measured using MTT and Western Blot assays, respectively. Pharmacokinetic studies and efficacy against infection were performed using a rabbit model, while ocular irritancy was assessed using the Draize test. The studies demonstrated that the antimicrobial activity of the moxifloxacin HCl was preserved following encapsulation into the nanofibers, while the downregulation of α-SMA was demonstrated using concentrations below the IC_{20} values (concentration required to decrease corneal fibroblast viability by no more than 20%). The pharmacokinetic study showed retention and sustained release of the moxifloxacin HCl over a 24-hour period, in contrast to equivalent eye drops which required four times daily dosing. Evidence of low level (according to the MMTS scale) irritation was detected for the nanofiber systems. Overall, the study has demonstrated that the dual drug-loaded nanofiber system shows potential for once daily dosing as an ocular insert for the treatment of corneal abrasion.

Keywords

Corneal abrasion, corneal infection, electrospinning, dual drug-loaded coaxial nanofibers, moxifloxacin, pirfenidone.

Graphical Abstract



1. Introduction

Corneal abrasion, which typically manifests as a small scratch on the anterior (corneal) section of the eye, may often require medical intervention in order to inhibit or prevent corneal infection and reduce the development of corneal fibrosis (Shahid and Harrison, 2013). The current treatment of this eye condition typically involves the topical administration of an antibiotic (eye drops or ointment), a cycloplegic agent, or a nonsteroidal anti-inflammatory drug with or without the use of artificial tears (Davids and White, 2020). However, disadvantages to the current approach include frequent dosing, extensive drug loss, burning eye sensations and blurred vision, all of which may lead to reduced patient acceptability (Dubald *et al*., 2018). Other treatment options include the application of eye patches such as pressure patching, bandage contact lenses and porcine collagen shields, again with concomitant safety and compliance issues associated with their use in injured eyes (Deka *et al*., 2020).

Electrospinning is a manufacturing technique that involves the application of a voltage to a polymer solution extruded from a needle, thereby overcoming the surface tension to produce a jet from which the solvent evaporates to produce micro- to nano-sized fibers (Williams *et al*., 2018). Such fibers may be single layered (monoaxial) or bilayered (coaxial) if a concentric needle assembly is used, each needle containing distinct polymer solutions. The use of electrospun nanofibers has received attention as a potential approach to drug delivery within the wound healing arena, not least because of the possibility of tailoring release while maintaining suitable mechanical flexibility and tensile strength (Aburayan *et al*., 2020). Fibrous mat networks formed from nanofiber systems have been studied as antibacterial dressings as well as wound healing and tissue regeneration matrices (Garg *et al*., 2015), while the ability of such systems to encapsulate a wide range of therapeutic agents (low molecular weight molecules through to biologicals and cellular therapies) alone and in combination is a further perceived advantage of these systems (Dziemidowicz *et al*., 2021).

A limited number of studies have involved the use of electrospun fibers for ocular applications, including the delivery of antimicrobials (Karatas *et al*., 2015; Baskakova *et al*., 2016; Sun *et al*., 2016), anti-glaucoma agents (Gagandeep *et al*., 2014; Lancina *et al*., 2017) and anti-inflammatory drugs (Mirzaeei *et al*., 2018). These studies have indicated that the electrospun fibers have *in vitro* and *in vivo* efficacy in, for example, inhibiting bacterial growth or reducing intraocular pressure, while providing prolonged drug release with low ocular irritation. The nanofibers systems described by Gagandeep *et al*., (2014), Sun *et al*. (2016) and Lancina *et al*. (2017) have been administered via external application under the eyelid or on the cornea as fibrous mats. These studies have shown that the judicious choice of fiber architecture and polymeric matrix into which the drugs are incorporated may lead to favorable release and efficacy, while the small size and flexibility of the fibers reduces discomfort and compromise of vision.

There has been very limited work to date exploring the use of electrospun fibers for the treatment of corneal scarring. Cejkova et al. (2016) suggested that cyclosporine-loaded fibers were more effective than equivalent eye drops for the treatment of corneal alkali burns; this was ascribed to the sustained drug release from the fibers compared to the eye drops that suffered from rapid corneal clearance.

Our previous *in vitro* work has investigated the use of electrospun nanofibers as a potential combination therapy approach to manage corneal abrasion (Tawfik *et al*., 2020). More explicitly, we have used coaxial systems to incorporate an antibiotic, moxifloxacin hydrochloride (HCl), and an anti-scarring agent, pirfenidone, in an outer layer of poly lactic-co-glycolic acid (PLGA) and inner layer of polyvinylpyrrolidone (PVP) respectively, with a view to generating a fibrous mat that could be self-administered by placing the construct underneath the eyelid. Our studies indicated a potentially favorable *in vitro* release profile of the two drugs, although *in vivo* studies are clearly required in order to assess the viability of the approach. More specifically, we noted that pirfenidone was completely released after 24 hours from the PLGA outer layer, while approximately 70% of moxifloxacin HCl was release over that same time from the inner PVP layer, with the incomplete release ascribed to entrapment inside the PLGA shell, as shown in Figure S1 (Tawfik et al., 2020). This release profile was considered to be potentially suitable for single daily dosing, with further refinement possible once the *in vivo* activity had been established.

The objective of the current work was therefore to assess the microbiological, anti-scarring (via downregulation of the α-SMA protein), and *in vivo* pharmacokinetic characteristics of the prototype formulation. In this manner it is intended that the potential applicability of the formulation, as well as the design improvements that may be required, will be established as the basis for a novel approach to the treatment of corneal abrasion.

2. Materials and Methods

2.1. Materials

HPLC grade water and acetonitrile (ACTN), pH 7.4 PBS tablets, agar powder, minimum essential medium eagle, MEM non-essential amino acid solution (100X), Dulbecco's PBS and thiazolyl blue tetrazolium bromide (MTT powder) were all obtained from Sigma Aldrich Company Ltd (Sigma Aldrich, Dorset, UK). Pirfenidone was purchased from Tokyo Chemical Industry UK Ltd (The Magdalen Centre, Oxford,

UK), while moxifloxacin HCl was purchased from Cambridge Bioscience Ltd (Munro House, Cambridge, UK). Tryptone soya broth (soybean casein digest medium,) and Iso-sensitest agar and were bought from Oxoid Limited (Hampshire, UK). Fluid thioglycollate medium was purchased from VWR International Ltd (VWR chemicals, Leicestershire, UK).

Bacterial strains were obtained from The National Collection of Type Cultures (NCTC, England, UK). Gram-positive *S. aureus* (NCTC 12981) and Gram-negative *P. aeruginosa* (NCTC 10662) were used in this study. Rabbit corneal fibroblasts (SIRC - Statens Seruminstitut Rabbit Cornea) was purchased from the American Type Culture Collection (ATCC® CCL-60™, Manassas, USA). Fetal Bovine Serum (FBS, qualified, heat inactivated, E.U.-approved, South America Origin), Penicillin-Streptomycin (10,000 units/mL for penicillin and 10,000 µg/mL streptomycin), GlutaMAX™ (100X) , sodium pyruvate 100 mM (100X), trypan blue solution (0.4%) and trypsin-EDTA (0.25% with phenol red) were obtained from Gibco (Fisher Scientific UK Ltd, Bishop Meadow Road Loughborough, UK). MES SDS running buffer (20X) and SDS-PAGE Bolt™ 4-12% bis-tris plus gels, 15 wells were purchased from Novex® (Life technology, Fisher Scientific UK Ltd, Bishop Meadow Road Loughborough, UK). Trans-Blot® Turbo™ 5X Transfer Buffer and TransBlot® Turbo™ mini-size low fluorescence polyvinylidene fluoride (LF PVDF) membrane were bought from Bio-Rad laboratories (USA). Marvel Original dried skimmed milk powder was purchased from Waitrose (London, UK). SuperSignal™ west femto maximum sensitivity substrate was obtained from Thermo Scientific (Rockford, US). Recombinant human transforming growth factor-β1 (TGF-β1) cytokine (ab50036), primary antibodies α-SMA (ab7817™) and β-Actin (ab8227™) with a molecular weight of 42 kDa for both antibodies, as well as secondary antibodies Goat Anti-Mouse IgG H&L (HRP) (ab205719™) and Goat Anti-Rabbit IgG H&L (HRP) (ab205718™), were purchased from Abcam (Cambridge, UK). Distilled water was generated by an ELGA Option 4 Water Purifier (Veolia Water Technologies, High Wycombe, UK). Vigamox® 0.5% w/v, were obtained from King Khalid University Hospital (Riyadh, Saudi Arabia). *S. aureus* bacterial strain (ATCC® 25923) was bought from ATCC (Manassas, USA). Mannitol Salt Agar (MSA) were purchased from Bacton Dickinson GmbH (Heidelberg, Germany). HPLC grade methanol was brought from BDH Chemical Ltd (Poole, England). Potassium dihydrogen orthophosphate was purchased from WINLAB laboratory chemicals reagents fine chemicals (Leicestershire, UK). Normal saline (0.9% NaCl solution) was obtained from Pharmaceutical Solutions Laboratory (Jeddah, Saudi Arabia). Ketamine hydrochloride (TEKAM®, 50 mg/mL) and xylazine (SETON®, 2% w/v) were bought from Hikma Pharmaceuticals (Amman, Jordan) and Laboratories Calier (Barcelona, Spain), respectively. Proparacaine hydrochloride ophthalmic solution (Alcaine®, 0.5% w/v) and tropicamide eye drop solution (Mydriacyl, 1% w/v) were purchased from Alcon Laboratories Inc. (Fort Worth, USA) and Alcon Eye Care UK Limited (Camberley, UK), respectively.

2.2. Animals

Thirty New Zealand male albino rabbits, weighing 3 to 4 kg each, were obtained from the experimental animal house care center at College of Pharmacy, King Saud University (Riyadh, Saudi Arabia). They were initially examined and screened for any pre-existing ocular conditions. All animals were maintained according to the guide for the care and use of laboratory animals approved by the center. All the rabbits were housed in clean cages (i.e. pathogen-free conditions). They were kept under standard laboratory conditions in 12 hours light and dark cycles at ambient temperature (25 ±

2°C), a pellet diet was given with water *ad libitum* and they were fasted overnight before the experiments. All animal studies were approved by the King Saud University Research Ethics Committee (approval no. KSU-SE-18-25). Anesthetics and artificial restraints were employed in this *in vivo* work.

2.3. Methods

2.3.1. Preparation of Coaxial Nanofibers

Coaxial fibers, blank and drug-loaded were prepared using a Spraybase® electrospinning instrument (Spraybase®, Dublin 2, Ireland) with the same electrospinning parameters mentioned in our previous paper (Tawfik *et al*., 2020) but under aseptic conditions. All plastic tubes and emitters (needles) of the Spraybase® instrument were autoclaved prior to their usage. In addition, the surrounding surfaces were wiped with 70% ethanol solution, along with the aluminum foil used for collecting the fibers. The yielded fibers were peeled by a pair of tweezers that were disinfected with 70% ethanol, and then kept in 50 mL sterilized centrifuge tubes. These fibers were later tested for sterility.

2.3.2. Sterility Testing of Blank and Drug-loaded Coaxial Nanofibers

Based on the British Pharmacopoeia (*Appendix XVI A. Test for Sterility,* 2019), two culture media can be used to test for sterility. Liquid thioglycollate (FTG) medium is used to assess the fibers for mainly anaerobic bacteria (such as *Clostridium sporogenes*), as well as aerobic bacterial strains (such as *S. aureus*), and was incubated at a temperature between 30ºC and 35ºC *(Appendix XVI A. Test for Sterility,* 2019). Soya-bean casein digest (SBC) medium was incubated at room temperature (between 20 and 25ºC). After preparing and autoclaving both media, a volume of 10 to 15 mL was added into airtight sterilized containers. Fiber weights of 1 to 2 mg were added and the systems were incubated for 14 days. A clear solution is an indication of the absence of microbial growth. In case of contamination, turbidity of the medium would usually occur in 3 days for bacterial contamination, while fungal growth would be expected to occur within 5 days *(Appendix XVI A. Test for Sterility,* 2019).

2.3.3. Determination of the Minimum Inhibitory Concentration (MIC) against Pathogenic Bacteria

To determine the MICs of moxifloxacin HCl and to check the antibacterial efficacies of pirfenidone against the Gram-positive *Staphylococcus aureus* (*S. aureus*) and the Gram-negative bacterium *Pseudomonas aeruginosa* (*P. aeruginosa*). *S. aureus* and *P. aeruginosa*, tests were conducted in iso-sensitest broth as described by Andrews (2001). Stock solutions of moxifloxacin HCl, pirfenidone and the combination of both drugs (1:1) were prepared by dissolving these drugs in sterile distilled water. A serial dilution of 11 concentrations (64 µg/mL to 0.03 µg/mL) of each drug and their combination (1:1) were prepared in 96-well plates (100 µL in each well), with one well for each series containing no drugs (bacterium only) as a growth control. 100 µL of a 1 x 10^6 colony-forming-unit/mL (CFU/mL) suspension of each microorganism in sterilized broth was then added into each well. This further diluted the drugs to be in the range of 32 µg/mL to 0.015 µg/mL. The plates were incubated at 37ºC for 24 hours, following the method of Andrews (2001). The turbidity of the solution (observed visually) is an indication of microbial growth with the MIC being defined as the lowest concentration at which no growth was observed.

2.3.4. Antibacterial Activity of the Drug-Loaded Coaxial Nanofibers using the Zone of Inhibition Assay

To evaluate the antimicrobial property of the electrospun fibers, the zone of inhibition test was performed based on the methods of Said *et al.* (2011) and Hilal Algan *et al.* (2016). The assessment was performed on the blank and drug-loaded coaxial fibers. *S. aureus* and *P. aeruginosa* were dispersed in sterile PBS (pH 7.4) at a concentration of 1 x 10^6 CFU/mL. Then approximately 2 mL of this bacterial suspension was distributed onto the surface of iso-sensitest agar plates. After drying, drug-loaded (weight equivalent to 70 µg moxifloxacin HCl) and blank (weight equivalent to the drug-loaded fiber) fibers were positioned in the center of the agar plate. A sterile disc that contained 50 µg moxifloxacin HCl was used as a positive control. The petri dishes were incubated at 37ºC for 24 hours with the diameters of the clear areas of 'no growth' around the formulations recorded in millimeters (mm). The results represent the mean (± SD) of at least 3 replicates.

2.3.5. *Rabbit Corneal Fibroblasts Sub-Culture Procedure*

Rabbit corneal fibroblasts (SIRC) have been used to assess the anti-fibrotic activity of pirfenidone and moxifloxacin HCl in free, combined and formulated forms. This cell line was propagated and passaged according to ATCC recommendations (*ATCC® CCL-60™*, 2019). A complete growth medium of minimum essential medium eagle adjusted by 1% penicillin-streptomycin, 1% MEM non-essential amino acid solution, 1% sodium pyruvate (100 mM), 1% GlutaMAX™ and 10% FBS was used. When the cell confluency reached 90%, the consumed medium was aspirated and the 75 cm² flask was rinsed with 5 mL Dulbecco's PBS to remove all traces of serum, as this contains a trypsin inhibitor which will interfere with the subsequent experimental procedure. A 3 mL of trypsin-EDTA (0.25%) solution was then added to the flask, to be incubated at 37□C and 5% CO_2 for 5 to 10 minutes. 5 mL of the complete medium was then added to neutralize the trypsin. The solution was then centrifuged at 1500 rpm for 5 minutes at 20□C. The supernatant was removed and a specified volume (5-10 mL) of the complete medium was added to the cell pellet to disperse it (*ATCC® CCL-60™*, 2019). After complete dispersion of the cells, cell counting was performed by using trypan blue solution (0.4%) to determine the number of cells to be transferred into well plates (for inhibitory concentration determination) or 75 cm² flasks (for sub-culturing).

2.3.6. In Vitro Determination of Drug Inhibitory Concentration (IC) against Fibroblasts

Rabbit corneal fibroblasts (SIRC) were used to assess the IC_{20} and IC_{50} of pirfenidone and moxifloxacin HCl. This cell line was propagated and passaged according to ATCC recommendations (*ATCC® CCL-60™*, 2019). After counting the cells, 40,000 cells per well were transferred to 48-well plates and incubated at 37□C and 5% CO_2. After 24 hours incubation, 50 to 60% confluency had been reached, which was sufficient for the subsequent experimental procedure (Tawfik, *et al.*, 2017). A 0.25 mL of test solution (pirfenidone, moxifloxacin HCl or both drugs, at a serial dilution range of 2.5 and 0.01 mg/mL) was added to each well and the cells were incubated for 24 hours (37□C and 5% CO_2). Untreated cells were used as controls for this experiment.

The MTT assay is a cell proliferation assay for determining the cell metabolic activity. An MTT solution (at a concentration of 0.5 mg/mL) was prepared by dissolving the MTT yellow powder in the culture media as described in Tawfik *et al.* (2017). After incubating the cells (exposed to the drugs) for 24 hours, the culture medium was

aspirated and the cells were supplemented with 0.25 mL of the MTT solution (in a dark biosafety cabinet) and incubated at 37°C and 5% CO_2 for 4 hours. The purple formazan crystals produced were solubilized by adding an equal volume (0.25 mL) of isopropanol to each well and gently shaking the plates for 30 minutes. Finally, the color intensity of each well was measured at 570 nm by a microplate reader and the percentage of cell viability was calculated relative to the untreated group. The results represent the mean (± SD) of at least 3 replicates.

2.3.7. Determination of Fibroblast Compatibility with the Blank and Drug-Loaded Coaxial Nanofibers

The initial growth and sub-culturing of the cells were similar to the previous section. After counting the cells, 80,000 cells per well were transferred to 24-well plates and incubated at 37°C and 5% CO_2. After 24 hours incubation, 50 to 60% confluency had been reached and the cells were treated for 24 hours with blank and drug-loaded coaxial fibers that were prepared aseptically in weights that contain 0.08 mg/mL pirfenidone. This was achieved be cutting square shaped pieces from the coaxial fiber mats into each well that contains 1 mL of the culture medium. These fibers weights were measured according to the previously reported drug loading in Tawfik *et al*. (2020). The drug loading was reported as 42 µg/mg and 23 µg/mg for pirfenidone and moxifloxacin HCl, respectively, owing to the initial twofold difference in the concentrations of pirfenidone (2% w/v) and moxifloxacin HCl (1% w/v) that were used in the preparation of the nanofibers (Tawfik et al., 2020). Therefore, a total weight of 1.8 mg of nanofibers were dissolved in 1 mL culture medium (i.e. equivalent to 0.08 mg/mL pirfenidone and 0.04 mg/mL moxifloxacin HCl concentrations) so as to ensure that the concentrations were below the threshold values corresponding to the IC_{20} for both drugs (see Section 3.3). The results were compared to the untreated control group, as well as the free and combined drugs that are in similar concentrations to the fibers.

After 24 hours incubation (37°C and 5% CO_2), the culture medium was aspirated and the cells were supplemented with 0.5 mL MTT solution (in a concentration of 0.5 mg/mL in a dark biosafety cabinet) and incubated at 37°C and 5% CO_2 for 4 hours. The yielded purple formazan crystals were solubilized by adding an equal volume (0.5 mL) of isopropanol to each well and gently shaking the plates for 30 minutes. Finally, the color intensity of each well was measured at 570 nm by a microplate reader and the percentage of cell viability was calculated relative to the untreated group. The results represent the mean (± SD) of at least 3 replicates.

2.3.8. Scanning Electron Microscopy (SEM)

The morphology of SIRC cells proliferated on the blank and drug-loaded coaxial nanofibers were demonstrated using a modified SEM method of Sun et al. (2014) and He et al. (2018). The fibers were incubated (37°C and 5% CO_2) with the cells for 24 hours, then were rinsed with Dulbecco's PBS then removed from the well and transferred to a new well, with no media. This material was kept in the incubator at 37°C and 5% CO_2 for a maximum of 5 minutes, in order to dry the fibers from the remaining buffer. The dried fibers were then adhered onto an SEM stub, using double sided carbon tabs (Agar Scientific, Stansted, UK). The prepared stub was then given a thin coating of gold (10 nM) in a Quorum Q150T Sputter Coater (Quorum Technologies Ltd. East Sussex, UK) in an argon atmosphere. The coated stub was

then transferred and imaged under FEI Quanta 200F (FEI company Ltd, Eindhoven, The Netherlands), at an acceleration voltage of 5 kV.

2.3.9. Protein Quantification for Western Blot Analysis

The anti-scarring effect was evaluated by exposing the SIRC cell lines to TGF-β1, in a concentration of 10 ng/mL, according to the methods of Chowdhury *et al.* (2013) and Stahnke *et al.* (2017). This was to mimic the immunological response after an injury, which will trigger the release of cytokines such as TGF-β. Therefore, inhibition of the expression of this protein is an indication of the anti-scarring effect of pirfenidone. The initial growth and sub-culturing of the cells were similar to the previous section. After cell counting, 160,000 cells per well were transferred to 6-well plates and incubated at 37°C and 5% CO_2. After 24 hours incubation, confluency had reached 50 to 60%, then the media was aspirated and the attached cells were treated with 2 mL serum free medium (i.e. without FBS) in order to starve the cells. After the overnight incubation, the serum free medium was aspirated and a 2 mL complete growth medium was added. Here, the medium will contain TGF-β1 (10 ng/mL), TGF-β1 (10 ng/mL) with pirfenidone (0.08 mg/mL), moxifloxacin HCl (0.04 mg/mL) and their 1:1 combination (pirfenidone 0.08 mg/mL and moxifloxacin HCl 0.04 mg/mL), as positive controls. These controls were compared with the blank and drug-loaded coaxial fibers in a total weight of 3.6 mg of nanofibers that were added to 2 mL culture medium which contain TGF-β1 (10 ng/mL), to maintain a concentration of 0.08 mg/mL for pirfenidone and 0.04 mg/mL for moxifloxacin HCl as previously explained in section 2.3.7. All these groups were tested against the untreated cell group, which contains complete growth media with no TGF-β1 for 48 hours incubation. The concentration of TGF-β1 that was used (10 ng/mL) to be incubated for 48 hours was based on the optimum conditions to stimulate the expression of extracellular matrix (ECM) proteins according to Narayanan *et al.* (1989).

After the incubation time point, cells were kept under ice. The medium was aspirated and the cells were rinsed with Dulbecco's PBS, then 0.25 mL of cold lysis buffer was added to each well for 10 minutes. The lysis buffer consists of NaCl (150 mM), Tris pH 7.5 (50 mM), EDTA (ethylene diamine tetra-acetic acid, 5 mM), NP40 (0.25% v/v), phosphate inhibitor (1 mL/ 100 mL total volume) and protease inhibitor (2 tablets in 100 mL total volume). The lysates were then transferred to 1 mL Eppendorf tubes to be centrifuged at 13,000 rpm and 4°C for 10 minutes. The supernatants were then transferred to new Eppendorf tubes for protein quantification. The bicinchoninic acid (BCA) protein assay was used to quantify the protein concentration in each sample. The protein standards and samples were prepared. Each standard concentration (50 µL) was mixed with lysis buffer (5 µL) and the BCA reagent (1 mL). However, the tested samples were prepared by mixing each sample (5 µL) with PBS (50 µL) and the BCA reagent (1 mL). The PBS was used as a diluent while the addition of the 5 µL lysis buffer with the protein standards was used to compensate the addition of 5 µL of the samples that already contain lysis buffer. All standards and samples were heated at 55°C for 30 minutes then transferred to 96-well plates to be measured under a microplate reader at a wavelength of 562 nm.

2.3.10. Determination of Anti-Scarring Proteins using the Western Blot Technique

After quantifying the protein concentrations, specified volumes of Laemmli sample buffer were added to each sample of the cell lysate protein and boiled (95°C) for 10

minutes, according to the methods of Chowdhury *et al.* (2013) and Stahnke *et al.* (2017). Equal quantities (25 µL) of each protein sample were then loaded in the wells of SDS-PAGE gel and separated at 100 V for 90 minutes using MES SDS running buffer (at a dilution of 1X). This running buffer consists of MES (2-(*N*-morpholino) ethanesulfonic acid), Tris Base (2-Amino-2-(hydroxymethyl)-1, 3-propanediol), SDS (Sodium dodecyl sulfate, also known as sodium lauryl sulfate) and EDTA at pH 7.4. The gel was transferred to PVDF membranes using transfer buffer (at a dilution of 1X). After transferring the membrane, it was washed with 1X TBST buffer, which consisted of Tris pH 9.5 (30 g), KCl (2 g), NaCl (80 g) and Tween 20 (1% v/v) in 1 L total volume, then the pH is adjusted to 7.4. The membrane was blocked with milk (5% w/v in 1X TBST) at room temperature for 2 hours. Finally, the membrane was incubated overnight at 4□C with the primary antibodies against α-SMA or β-Actin diluted in 5% milk, as described in Chowdhury *et al.* (2013) and Stahnke *et al.* (2017) in the recommended concentrations by Abcam (Cambridge, UK).

After the overnight incubation, the membrane was washed 3 times quickly with TBST, followed by 3 long washes (10 minutes each). Then the membrane was incubated (at room temperature for 1 hour) in the HRP conjugated secondary antibody diluted in 5% milk in the recommended concentration. Secondary antibody, goat anti-mouse or goat anti-rabbit, was used for the membrane which was incubated with α-SMA or β-Actin, respectively. After that, each membrane was washed 3 times quickly with TBST, followed by 3 long washes, as described by Chowdhury *et al.*, 2013 and Stahnke *et al.*, 2017. Finally, specific antigen-antibody complexes were developed by using SuperSignal™ west femto maximum sensitivity substrate and the chemoilluminescence was detected using a Syngene GeneGnome Bio imaging system (Syngene, Cambridge, UK). Optical density of each sample band was normalized to the corresponding β-Actin band, which was further quantified using ImageJ software, where the band area of the α-SMA for each group was divided by its corresponding band area of the β-Actin for the same group, following the method of Stahnke *et al.* (2017).

2.3.11. In Vivo Ocular Pharmacokinetic Study using the Drug-Loaded Coaxial Nanofibers

Drug loading of the coaxial nanofibers was estimated as 40 µg/mg for pirfenidone and 21 µg/mg for moxifloxacin HCl. Pharmacokinetic studies were performed following the application of 11.90 mg of the coaxial fibers, equivalent to 250 µg of moxifloxacin HCl and 476 µg of pirfenidone in one dose. For the test control, 50 µL from the commercially available moxifloxacin HCl eye drops (0.5% w/v) will contain an equivalent amount of moxifloxacin HCl (250 µg) to the fibers, while an amount of pirfenidone equivalent to that of the fibers (476 µg) was added to the 50 µL moxifloxacin HCl solution. The fibers were applied under the lower eyelid, adjacent to the conjunctival sac, of the rabbits' eye.

To assess ocular bioavailability, drug concentration in aqueous humor after a single topical ocular dose of drug-loaded coaxial fibers in rabbits was compared to a single dose of moxifloxacin HCl (0.5% w/v) and pirfenidone eye drop, based on the method described by Kalam (2016). Six rabbits were divided into two groups, each containing three animals (n=3), in which one group (Group 1) received drug-loaded coaxial fibers while the second group (Group 2) received 50 µL of the combined drugs in solution

form. Approximately 11.9 mg of the coaxial fibers were inserted under the lower eyelid of the left eyes of all rabbits in Group 1. All the rabbits were placed in restraining boxes where they could freely move their heads throughout the study period. One hour after dosing, rabbits were anesthetized by intravenous ear vain injection of a mixture of ketamine HCl (15 mg/kg) and xylazine (3 mg/kg). One drop of proparacaine HCl (0.5% w/v) and one drop of tropicamide (1% w/v) were inserted into the treated eye. Then, 50 µL of aqueous humor was withdrawn using a 29-gauge insulin syringe needle at an angle of 45º through a partial thickness limbal incision at different time points (1, 2, 4, 6, 12 and 24 hours) from the treated eyes of all rabbits of the respective groups. For each time point, aqueous humor samples were collected from the treated eyes of the rabbits (n=3).

The collected samples were transferred into centrifuge tubes and analyzed by the developed ultra-performance liquid chromatography (UPLC) method (shown in the Supplementary Information section) at a UV-detection wavelength of 310 nm in order to quantify the drugs. The pharmacokinetic parameters were determined by PK-Solver software (Nanjing, China), as described in Zhang *et al*. (2010). The animals from both groups were sacrificed after the experiment in a humane way, by an intraperitoneal injection of pentobarbitone sodium at a concentration of 60 mg/mL and a dose of 60 mg/kg of body weight.

2.3.12. In Vivo Infection Inhibition Study using Drug-Loaded Coaxial Nanofibers

Twelve rabbits were divided into four groups (n=3 in each). Gram-positive *S. aureus* was instilled in one eye at a concentration of $1x10^6$ CFU/mL to cause an infection. Before instillation, eye swabs were taken from the eyes to check for any microorganism growth i.e. previous infection on MSA plates. After 24 hours of post *S. aureus* instillation, eye infections were observed visually as redness. Eye swabs, taken from the infected eyes, were checked for any microorganism growth on pre-sterilized MSA plates followed by treatment initiation. To the first group of infected eye rabbits, moxifloxacin HCl eye drops (0.5% w/v) were applied 4 times daily. The infected eyes of the second group were left without further treatment, as the untreated control group. In the third and fourth groups of rabbits, the blank and drug-loaded coaxial fibers were applied under the lower eyelid of the infected eyes. An equivalent weight of the drug-loaded fibers to the moxifloxacin HCl eye drop dose was measured and inserted under the eyelid (Group 3). The blank fibers (in a similar weight as the drug-loaded fibers) was also inserted under the eyelid of the rabbits (Group 4). Treatment was given daily at the same time and continued for 7 days. The animals from all groups were kept under investigation for 5 days posttest then sacrificed on day 12 after the infection. Throughout the whole experiment, photographs of the cornea were taken at designated time points.

2.3.13. In Vivo Ocular Irritation Study of the Blank and Drug-Loaded Coaxial Nanofibers

Eye irritation was measured in rabbit eyes based on the Draize test (Draize *et al*., 1944, Luechtefeld *et al*. 2016 and Kalam, 2016). Twelve rabbits were divided into two groups each containing six rabbits (n=6). Equivalent weights of drug-loaded fibers in one group

of rabbits and similar weights of blank fibers in the other group were placed under the lower eyelid of the left eyes. The right eyes of both groups were instilled with sterile normal saline to serve as control. The fibers were given once daily for 7 days and the rabbits' eyes were observed visually throughout the whole study. The eye irritation level was evaluated by the animal discomfort, signs and symptoms in the conjunctiva, cornea, and eyelids according to the scoring guidelines system for ocular irritation testing mentioned in Table S4 that is shown in the Supplementary Information section (Draize *et al*., 1944, Higuchi *et al*., 2016 and Kalam, 2016). The results of the obtained weighted scores is shown in Table S5 in the Supplementary Information section The maximum mean total score (MMTS) is the sum of all scores obtained for the cornea, iris and conjunctiva and the overall irritation scores will be related to Table 1. Scores of 0 are assigned for each parameter if the cornea, iris or conjunctiva is normal. The animals from both groups were kept under investigation for 5 days post-test after the treatment with the blank and drug-loaded fibers, then sacrificed on day 12 (in the humane way described previously).

Table 1: Kay and Calandra (1962) classification of ocular irritation scores.

Maximum Mean Total Score (MMTS)	Irritation Classification
0.0 - 0.5	None
0.6 - 2.5	Practically none
2.6 – 15	Minimally
15.1 – 25	Mildly
25.1 – 50	Moderately
50.1 – 80	Severely
80.1 – 100	Extremely
100.1 – 110	Maximally

2.3.14. Statistical Analysis

Mean comparison was performed using one-way analysis of variance (ANOVA) using GraphPad Prism® statistical software, followed by Tukey's multiple comparison post hoc tests and $P < 0.05$ was taken as a criterion for a statistically significant difference. All results are expressed as mean ± SD.

3. Results and Discussion

3.1. *Sterility testing and Determination of the Minimum Inhibitory Concentration (MIC) against Pathogenic Bacteria*

After fabricating the blank and drug-loaded fibers under aseptic conditions, sterility testing was performed according to the British Pharmacopeia (*Appendix XVI A. Test for Sterility,* 2019). The lack of turbidity and fungal growth for the blank and drug-loaded fibers after 14 days of incubation was sufficient to confirm the sterility of these fibers, hence these fibers were further tested using *in vitro* and *in vivo* models. An image of the sterility test for the tested fibers after 14 days, as well as an intentionally

contaminated medium (to confirm the capacity of the medium to support microbial growth) is shown in Figure S2 in the Supplementary Information section.

The minimum inhibitory concentrations (MICs) for both drugs, individually and in combination (1:1), against the Gram-positive bacterium *Staphylococcus aureus (S. aureus)* and the Gram-negative bacterium *Pseudomonas aeruginosa (P. aeruginosa)* were determined initially. This is required in order to evaluate the antibacterial efficiency of moxifloxacin HCl and pirfenidone and to ascertain whether there is any synergistic or resistance effect resulting from their combination.

The MICs was measure in a concentration range of 0 to 32 µg/mL. The presence of turbidity indicated bacterial growth. The MIC values of moxifloxacin HCl were 0.125 µg/mL and 4 µg/mL against *S. aureus* and *P. aeruginosa,* respectively, in which they were similarly determined (same MIC values) in two different experimental days to validate these values. This suggested a lower susceptibility of *P. aeruginosa* to moxifloxacin HCl compared to *S. aureus*. This finding was in agreement with Mudgil and Pawar (2013), who reported a higher susceptibility of *S. aureus* to moxifloxacin loaded into PLGA nanosuspension formulation than *P. aeruginosa* after 12- and 24-hours incubation. In addition, these values were within ± one two-fold dilution of the expected MIC reported by Andrews (2001), who stated moxifloxacin HCl MIC values of 0.06 µg/mL and 2 µg/mL against *S. aureus* and *P. aeruginosa*, respectively. Pirfenidone demonstrated no activity against either bacterial strains, while in combination showed similar MIC values to moxifloxacin HCl alone; this indicates a lack of synergistic activity against *S. aureus* and *P. aeruginosa*. Figure S3 illustrates the MIC assay outcomes.

3.2. Antibacterial Activity of the Drug-Loaded Coaxial Nanofibers using the Zone of Inhibition Assay

The ability of the drug-loaded nanofibers to inhibit the growth of bacteria was assessed using the zone of inhibition test, in a fiber size equivalent to a dose of 70 µg of moxifloxacin HCl considering the release profile of these fibers (Tawfik et al., 2020), compared to the blank fibers (negative control) and a moxifloxacin HCl disc at a dose of 50 µg (positive control) against *S. aureus* and *P. aeruginosa.* After 24 hours incubation, well-defined zones of inhibition for the drug-loaded nanofibers and the moxifloxacin HCl discs were observed (Figure S4).

The zone of inhibition diameters for the drug-loaded nanofibers were 45 mm (± 1 mm) against *S. aureus* and 28 mm (± 1 mm) for *P. aeruginosa* compared to 42 mm (± 1 mm) and 26 mm (± 1 mm) for moxifloxacin HCl discs, respectively. The blank fibers exhibited no effect against either bacterial strain. This result indicated that moxifloxacin HCL was successfully released and retained its antibacterial activity against the two bacterial pathogens. In addition, the presence of pirfenidone and the polymers showed no effect on the antibiotic potency of moxifloxacin HCl, nor did they exhibit any intrinsic antibiotic effect of their own.

Previous studies have reported the antibacterial efficacy of moxifloxacin-loaded electrospun fibers against different bacterial strains; for example, Shawki *et al*. (2010) demonstrated the antibacterial efficacy of dextran nanofibers loaded with moxifloxacin against both *S. aureus* and *E. coli*. Toncheva *et al*. (2012) was able to demonstrate antibacterial activity of Poly-l-Lactide-Co-d, l-Lactide (coPLA) and a blend of coPLA and PEG-containing moxifloxacin hydrochloride against *S. aureus.* Cheng *et al*. (2015) evaluated the antibacterial activity of moxifloxacin-loaded into a chitosan and PEO

blend (9:1) which was active against *S. aureus* and *E. coli*. A more recent study by Giram *et al*. (2018) reported the antimicrobial efficacy of moxifloxacin-loaded into Eudragit L-100 against *S. aureus* and *E. coli*. Overall, therefore, the findings reported here are compatible with previous studies that have indicated that moxifloxacin HCl is released from nanofibers over a 24 hour period and retains its efficacy against model pathogenic bacteria.

3.3. In Vitro Determination of Inhibitory Concentration (IC) against Fibroblasts

The following set of experiments were designed so as to assess the ability of the fibers to downregulate expression of proteins (in this case α-smooth muscle actin) that are associated with scar formation. In order to achieve this, it was necessary to first determine the concentration of both drugs that may be applied without causing significant damage (Section 3.3) to the constituent fibroblasts and then to assess the biocompatibility of the loaded fibers with the fibroblasts (Section 3.4).

Growth inhibitions of the rabbit corneal fibroblasts against solutions of moxifloxacin HCl, pirfenidone and their combination were assessed and the growth inhibitory concentrations (IC) 20% (IC_{20}) and 50% (IC_{50}) determined. Corneal fibroblasts (also known as keratocytes) were selected as they reside in the corneal stromal layer, which represent approximately 85% of the corneal thickness. These cells respond to corneal injury through the release of inflammatory mediators such as TGF-β that may be readily quantified (Chaurasia *et al.* 2015); this therefore allows determination of the effectiveness of the drug-loaded nanofibers in downregulating ECM proteins that could be triggered as a response to inflammatory mediator release following corneal abrasion (described in Section 3.5). Here, fibroblast viability was measured as a mean of assessing (unencapsulated) drug toxicity, with 80% cell viability (which represents the IC_{20}) being the maximum inhibition that can be considered acceptable at this stage of the development process.

The SIRC cell line was successfully cultured according to ATCC recommendations (*ATCC® CCL-60™*, 2019). Figure 1 shows the cell viability after 24-hour exposure to the drugs alone. Moxifloxacin HCl concentrations of 0.04 mg/mL or less exhibited greater than 80% cell viability, with higher concentrations leading to increased cell death below this threshold figure. Pirfenidone was less toxic than moxifloxacin HCl, with cell viability of $\geq$ 80% seen with concentrations up to 0.31 mg/mL. The effects of the combination followed the pattern of the moxifloxacin HCl alone, indicating that there was no interaction between the two drugs with respect to cell viability.

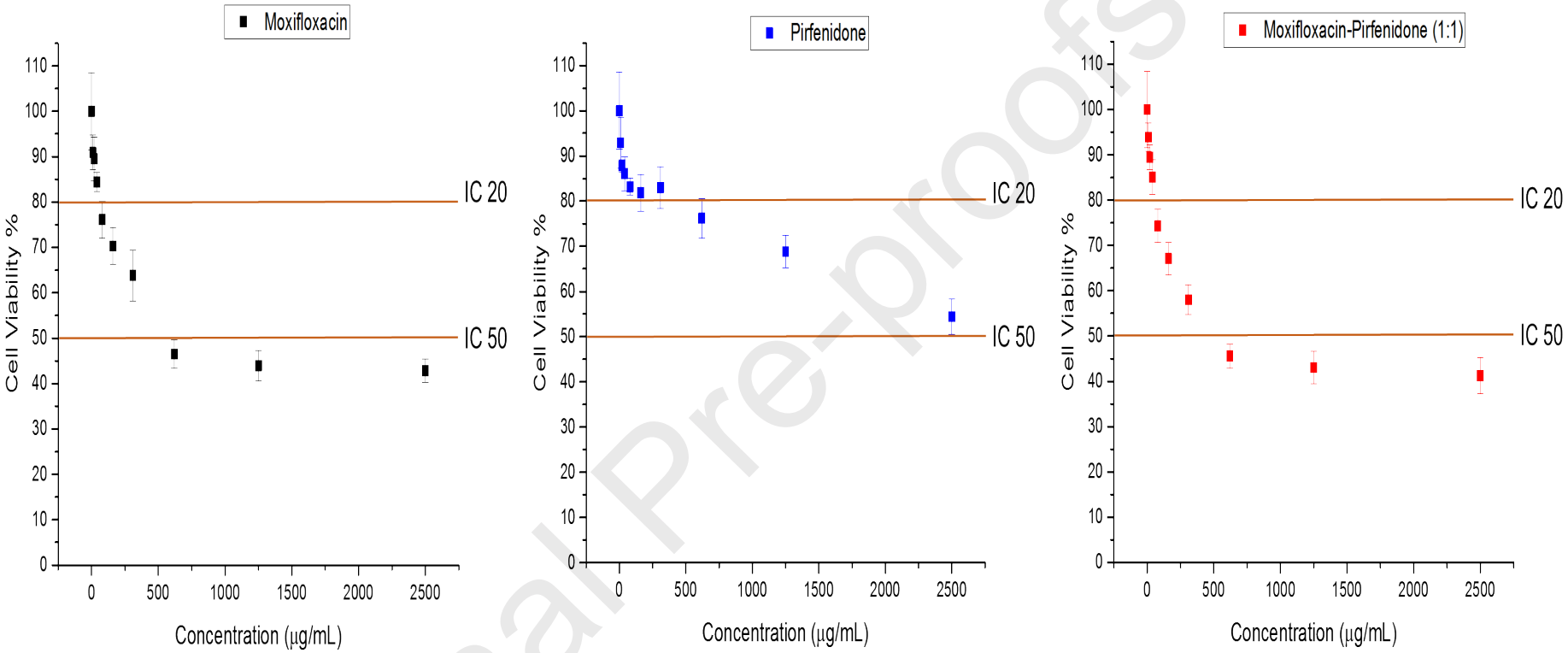


Figure 1: IC20 and IC50 determination after 24-hour exposure to different concentrations of the free drugs showing the dose response curves of the mean ± SD (n=3). The left curve represents moxifloxacin HCl, the middle curve represents pirfenidone, and the right curve represents moxifloxacin HCl and pirfenidone in 1:1 combination. After 24-hour cell-exposure, moxifloxacin HCl concentrations of 0.04 mg/mL or less showed greater than 80% cell viability, while pirfenidone showed less toxicity than moxifloxacin HCl, with cell viability of ≥ 80% was shown with concentrations up to 0.31 mg/mL. The results represent the mean (± SD) of n=3.

3.4. Determination of Fibroblast Compatibility with the Blank and Drug-Loaded Coaxial Nanofibers

Fibroblast cell exposure to the drug-loaded fibers (equivalent to 0.08 mg/mL of pirfenidone and 0.04 mg/mL of moxifloxacin HCl) was studied in order to compare the toxicity of the fibers to the equivalent solutions. Figure 2 indicates that the drug-loaded coaxial nanofibers led to a lower reduction in cell viability (8.75%) compared to the free pirfenidone (14.90%), moxifloxacin HCl (17.50%) and the 1:1 combined drugs (19.75%) after 24-hour cell-exposure, while the blank fibers showed no reduction in the cell viability. This is probably due to the relative slow drug release rates that enabled the cells to survive and proliferate compared to the cells that were exposed to the drugs in solution form.

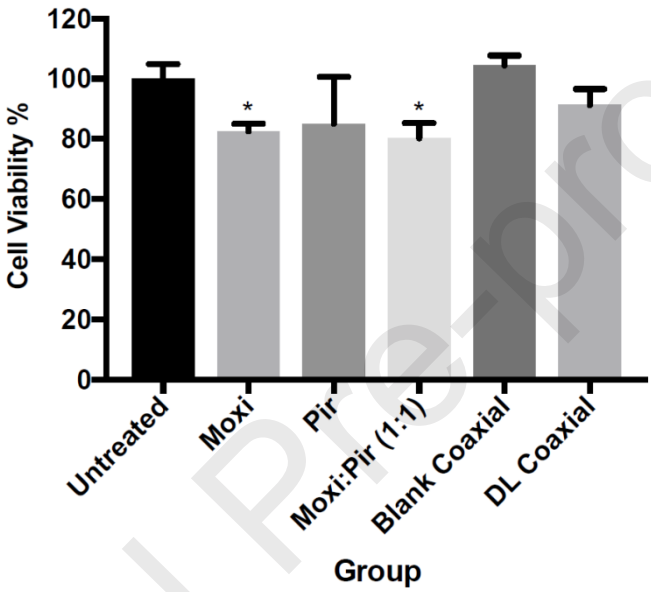


Figure 2: Cell viability (%) after 24-hour cell-exposure to free drugs, blank and drug-loaded coaxial nanofibers in equivalent concentrations of 0.08 mg/mL (80 µg/mL) for pirfenidone and 0.04 mg/mL (40 µg/mL) for moxifloxacin HCl. The results represent the mean ± SD (n=3) and P < 0.05. After 24-hour cell-exposure, drug-loaded coaxial fibers reduced the cell viability by approximately 9% compared to the reduction of 15 to 20% of the free drugs, while the blank fibers showed no reduction in the cell viability.

SEM was utilized to demonstrate the morphology of SIRC cells proliferated on the fibrous network of the blank and drug-loaded coaxial nanofibers after 24-hour exposure; this is shown in Figure S5 in the Supplementary Information section. It was observed that both fiber systems promoted cell attachment and spreading, with cells embedded into the fibrous network gaps forming a confluent layer. This is an indicator of good cell compatibility. A similar SEM observation was demonstrated by Sun *et al.* (2014) study on PLGA fibers cultured with human dermal fibroblasts and He *et al.* (2018) study on PLGA/PVP coaxial fibers cultured with mouse bone fibroblasts.

3.5. Determination of Anti-Scarring Proteins using the Western Blot Technique

ECM proteins, including α-smooth muscle actin (α-SMA), may be overexpressed in response to injury and can therefore be used as a marker for the propensity for scarring, hence the levels of α-SMA were evaluated as a function of drug exposure. Pirfenidone in particular has been shown to inhibit scar formation following corneal

abrasion, especially in deeper wounds (Bukowiecki *et al*., 2017; Ljubimov and Saghizadeh, 2015), and hence downregulation of α-SMA may be used as an indicator of the potential for the inhibition of scarring.

Initially, the cells were deprived of nutrients via overnight incubation in serum free medium so as to allow the cells to synchronize to the same growth cycle. In addition, this procedure facilitates the avoidance of interactions between serum components and reagents or expressed proteins during the experimental procedure (Pirkmajer and Chibalin, 2011). The cells were then exposed to TGF-β1 for 48 hours to induce expression of ECM proteins. After 24 hours incubation with TGF-β1, the cells were exposed to various treatments including solutions of pirfenidone, moxifloxacin HCl alone and in combination, as well as blank and drug-loaded coaxial nanofibers. α-SMA expression was evaluated using Western blot analysis, using β-actin as the experimental control.

Figure 3 illustrates the enhanced expression of α-SMA after 48-hour exposure to TGF-β1 compared to the untreated group. The band intensities also demonstrate the reduction of α-SMA expression after treatment with solutions of pirfenidone, the combination of pirfenidone and moxifloxacin HCL and, interestingly, moxifloxacin HCl alone. A similar anti-scarring effect of moxifloxacin had previously reported by Chen *et al.* (2013) on human corneal fibroblasts that showed that the drug was able to decrease the expression of α-SMA after TGF-β1 stimulation and inhibit TGF-β-induced fibroblast-to-myofibroblast differentiation. The drug-loaded coaxial nanofibers exhibited a significant ($p < 0.05$) decrease in α-SMA expression which was similar to the positive controls (free drugs) and the blank fibers compared to the TGF-β1 only group. Surprisingly, the blank coaxial fibers were able to down-regulate the expression of α-SMA ($p < 0.05$) compared to the TGF-β1 only group. This might be due to the presence of the polymers and will require further investigation. Overall, the study has suggested that the drug-loaded coaxial nanofibers led the reduction of the up-regulated α-SMA at a level similar to that of the drugs in solution form. A full image of the Western blot bands is available in Figure S6.

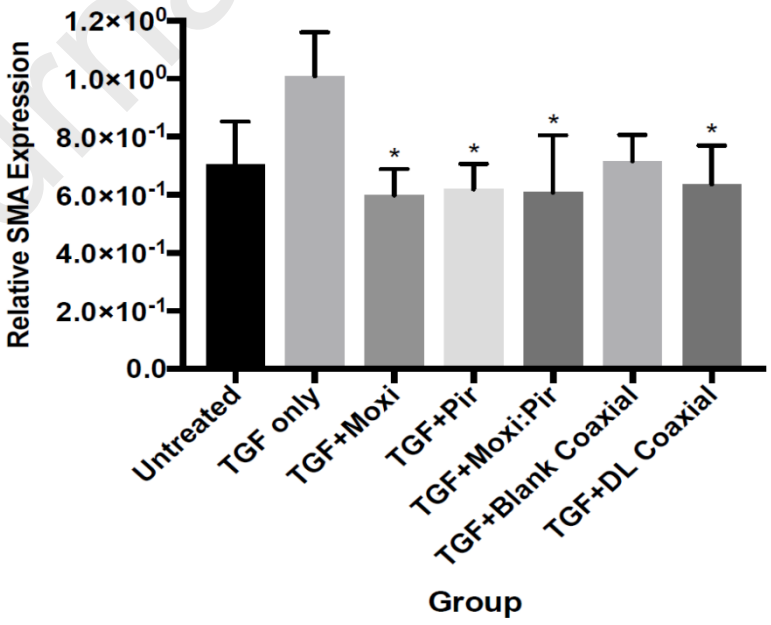


Figure 3: Western blot quantification using the band intensities of α-SMA (protein of interest) over the corresponded β-Actin (experimental control). The bands were obtained after 48-hour exposure to TGF-β1 alone or in combination with the free drugs, blank or drug-loaded coaxial nanofibers in equivalent concentrations of 40 µg/mL compared to the untreated group. B: blank and DL: drug-loaded. The results represent the mean ± SD (n=3) and P < 0.05 for all groups in comparison to the TGF-β1 only group. The drug-loaded coaxial nanofibers showed a similar reduction in the expression of α-SMA to the free drugs moxifloxacin HCl (Moxi), pirfenidone (Pir) and the drug combination (Moxi:Pir) in 1:2 ratio.

3.6. In Vivo Ocular Pharmacokinetic Study of the Drug-Loaded Coaxial Nanofibers

A pharmacokinetic study of the drug-loaded coaxial fibers in New Zealand rabbit eyes was performed in order to compare the loaded nanofibers with the corresponding free drugs. An UPLC standard curve for both drugs were developed in order to analyze the withdrawn samples from the rabbit aqueous humor (the method development is available in the Supplementary Information Section S7). Figure 4 illustrates the aqueous humor concentration of moxifloxacin HCl and pirfenidone after topical application of the drug-loaded coaxial nanofibers or the drug solution.

Solutions of the combined drugs was measured up to 6 hours after ocular instillation; thereafter, the concentration of both drugs was not measurable at the 12-hour time point, as it can be seen in Figure 4 (a) and (b). This indicates fast pre-corneal loss of both drugs when administered in solution form. However, both drugs were quantified in the aqueous humor samples up to 24 hours after the topical application of the drug-loaded coaxial nanofibers. Prolonged release of moxifloxacin has also been reported by Warsi *et al*. (2012) using nanoplexes, hence there is precedent for controlled ocular release of the drug using nanofabricated systems. Similarly, studies by Kalam (2016) involving dexamethasone loaded into chitosan nanoparticles for ocular delivery and Gagandeep (2014) on timolol maleate and dorzolamide hydrochloride-loaded PVA and PCL nanofibers showed similar trends.

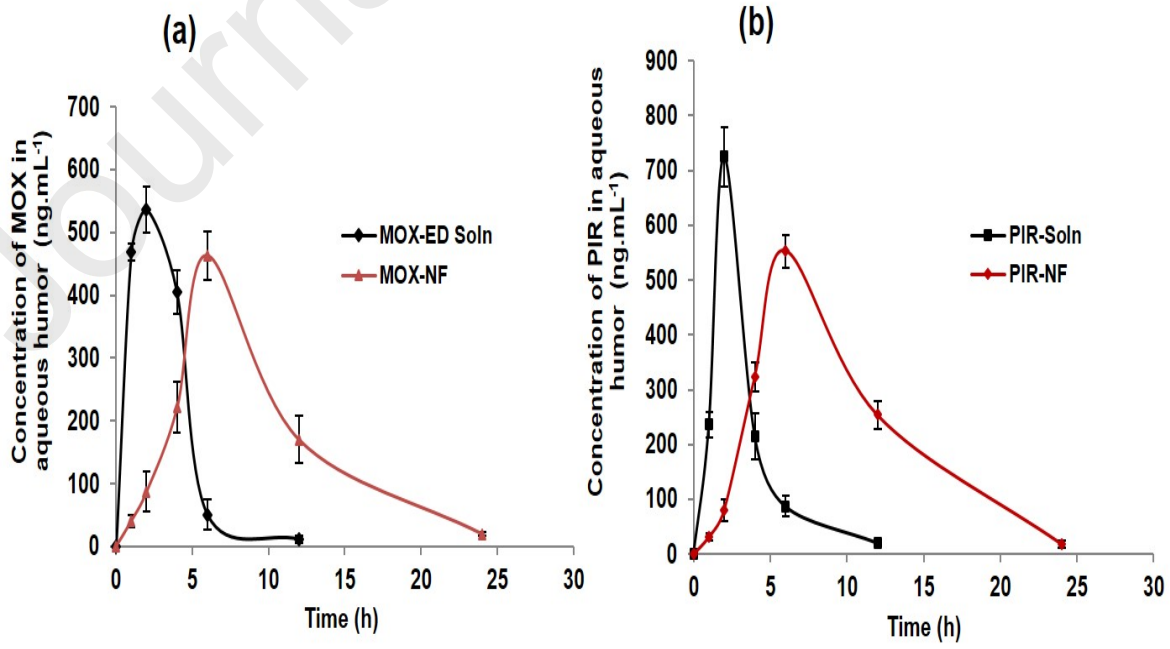


Figure 4: The aqueous humor concentrations of (a) moxifloxacin HCl and (b) pirfenidone versus time profiles following topical ocular application of both drugs either in solution form (ED-Soln) or coaxial nanofibers (NF) to the rabbits. The results represent the mean ± SD (n=3). The topical application of the combined drugs in solution form was measured up to 6 hours compared to 24 hours for the drug-loaded coaxial fibers.

Additionally, Table 2 summarizes the results using different pharmacokinetic parameters. The drug-loaded coaxial fibers showed a significantly ($p < 0.05$) higher ocular bioavailability of both drugs than the solution form. The $AUC_{0\text{-}24h}$ of moxifloxacin HCl and pirfenidone from the drug-loaded coaxial fibers was greater than the solution form, with 1.77-times and 2.49-times enhanced values, respectively. The $t_{1/2}$ of moxifloxacin HCl and pirfenidone was significantly ($p < 0.05$) higher in the fibers compared to the solution form, with 2.34-times and 1.43-times higher values, respectively. The C_{max} of moxifloxacin and pirfenidone from the fibers was lower than the corresponding values for the solutions (0.86- and 0.76-times respectively), reflecting the alteration in the timescale over which the drugs are released from the two systems, in turn reflected by the observed T_{max} values.

Therefore, the results suggest that the drug-loaded coaxial fibers have the potential to be applied in a single daily dose, with both prolonged release and enhanced bioavailability demonstrated over a 24 hour period.

Table 2: Ocular pharmacokinetic parameters of moxifloxacin HCl and pirfenidone in aqueous humor after topical application of the drug-loaded coaxial fibers and both drugs in solution form. The results represent the mean ± SD (n=3) for each time point.

Pharmacokinetic parameters	Moxifloxacin HCl in solution form	Moxifloxacin HCl in the fibers	Pirfenidone in solution form	Pirfenidone in the fibers
$t_{1/2}$ (h)	1.7 ± 0.2	3.9 ± 0.1	2.5 ± 0.3	4.0 ± 0.3
T_{max} (h)	2.0 ± 0.0	6.0 ± 0.0	2.0 ± 0.0	6.0 ± 0.0
C_{max} (ng/mL)	537 ± 37	464 ± 39	725 ± 55	553 ± 29
$AUC_{0\text{-}24h}$ [ng/(mL.h)]	2316 ± 207	4111 ± 613	2166 ± 172	5410 ± 425
$AUC_{0\text{-}inf}$ [ng/(mL.h)]	2344 ± 224	4221 ± 631	2241 ± 165	5511 ± 468
$AUMC_{0\text{-}inf}$ [ng/(mL.h^2)]	7331 ± 1260	37595 ± 6127	8163 ± 652	49278 ± 5755
$MRT_{0\text{-}inf}$ (h)	3.1 ± 0.3	8.9 ± 0.2	3.6 ± 0.1	8.9 ± 0.3
Vz/F [ng/(ng/mL)]	284 ± 20	364 ± 48	778 ± 142	449 ± 11
Cl/F {ng/[(ng/mL)/h]}	107 ± 10	60 ± 9	213 ± 16	87 ± 7

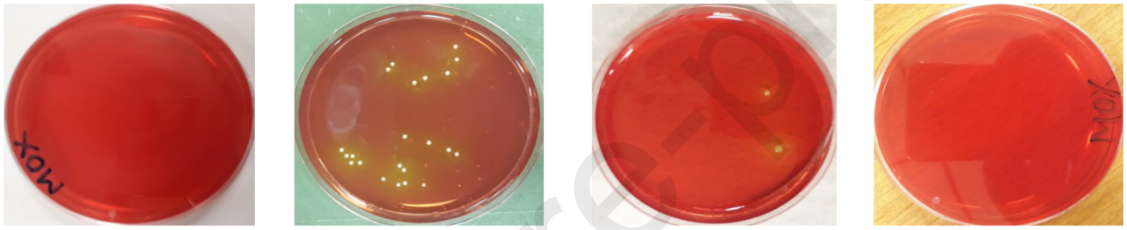
3.7. In Vivo Infection Inhibition Study of the Drug-Loaded Coaxial Nanofibers

An infected rabbit eye model was utilized whereby each infected eye was treated with a once daily dose of the drug-loaded fibers compared to 4-time daily doses of moxifloxacin HCl eye drops. Prior to the treatment, eye swabs were taken in order to check for the existence of a previous infection as shown in Figure 5 (a; pre-infection). One eye was infected using a *S. aureus* bacterium. After 24 hours post infection, the infection was visually observed via the presence of eye redness. In addition, eye swabs

were taken and the bacterial growth checked on pre-sterilized MSA plates (Figure 5 (b) post infection), after which the treatment was initiated.

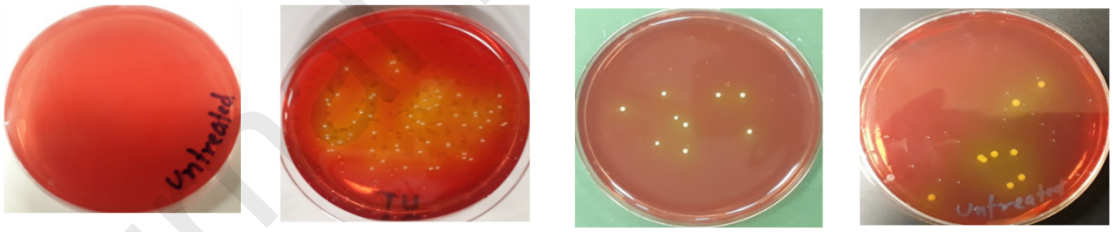
The first group of rabbits received the marketed moxifloxacin HCl eye drop (0.5% w/v), which was applied 4 times daily into the infected eyes and photographs of the swab test were taken at different time points, as shown in Figures 5 - Group A. The results showed a complete absence of bacterium colonies after 7 days of treatment, with evidence of infection still present after 3 days. The group of rabbits that received once daily drug-loaded coaxial nanofibers under the lower lid (third group of rabbits, Figure 5 - Group C) showed the same pattern, indicating equivalence of efficacy. For the untreated group of rabbits (Figure 5 - Group B) and the group which was treated with blank nanofibers (Figure 5 - Group D), bacterial colonies were still detected after 7 days of infection. Photographs of the rabbit eyes are shown in Figure S10 the Supplementary Information section. These results indicated that a single daily dose of the drug-loaded coaxial nanofibers has an equivalent efficacy to 4-time daily dose of moxifloxacin HCl eye drops.

A. MOX-ED treated group (eye swab)



(a) Pre infection | (b) Post infection | (c) Post treatment (3 days) | (d) Post treatment (7 days)

B. Untreated group (eye swab)



(a) Pre infection | (b) Post infection | (c) Post treatment (3 days) | (d) Post treatment (7 days)

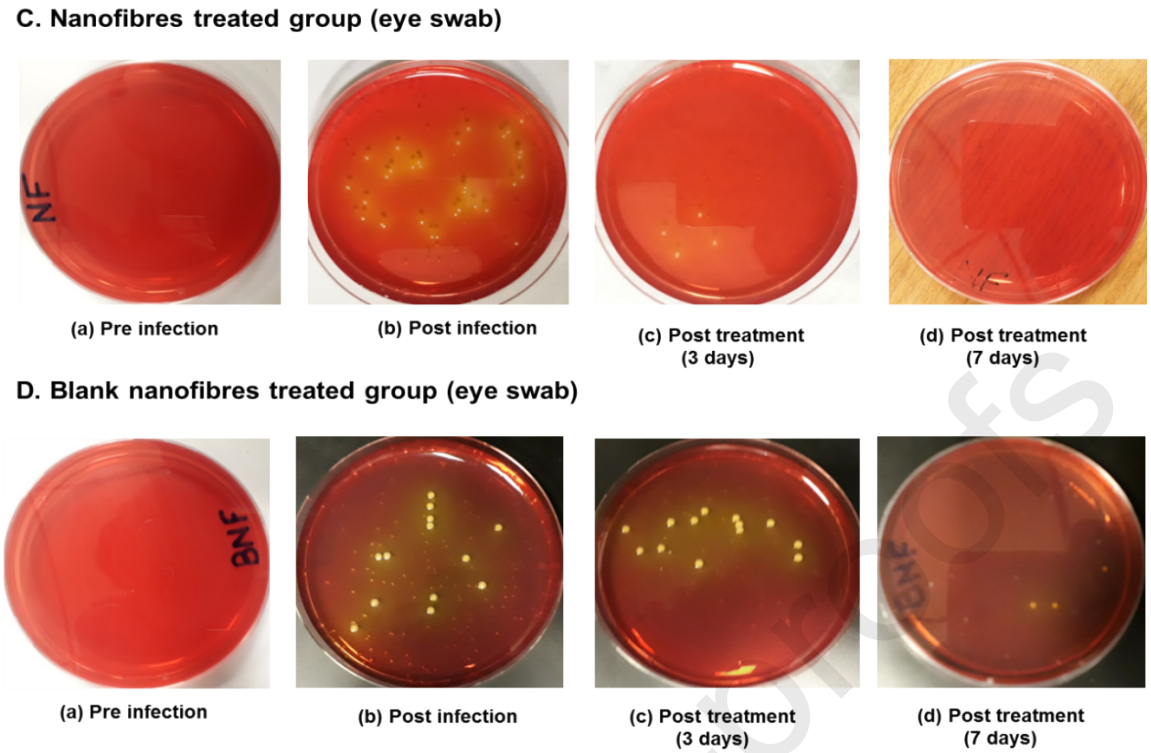


Figure 5: Eye swabs for the growth of S. aureus on MSA plates; (a) pre infection (no bacterial growth), (b) 24-hour post infection, (c) 3-day post treatment and (d) 7-day post treatment. A: Moxifloxacin HCl eye drops, B: untreated control group, C: drug-loaded coaxial nanofibers, D: blank coaxial nanofibers. After 7 days post infection, the group of rabbits that were treated with single daily dose of the drug-loaded coaxial fibers showed an absence of bacterium colonies similarly to the 4 daily doses of moxifloxacin HCl eye drops, while the untreated group and the blank fibers treated group showed a presence of bacterium colonies.

3.8. In Vivo Ocular Irritation Study In Vivo Ocular Irritation Study of the Blank and Drug-Loaded Coaxial Nanofibers

It was necessary to evaluate the blank and drug-loaded coaxial nanofibers irritability to the anterior segments of the eye using the standard Draize test (Draize et al., 1944). This is performed by visually observing the cornea, iris and conjunctiva of the rabbits after inserting the fibers under the lower eye lid of one eye once daily for 7 days. Based on the symptoms that may occur on the eye that has been treated with the nanofibers, such as redness, chemosis or presence of any discharge, it is possible to score the rabbit eye according to the scores' grading classification that is described in Table S4 in the Supplementary Information section. The scores obtained for this irritation study, including the signs of discomfort, are illustrated in Table S5 in the Supplementary Information section for both the blank and drug-loaded coaxial nanofibers. As a result of the single application of the nanofiber, a slight irritation was shown in a few rabbit eyes. A minor mucoidal discharge (grade 2) and some watery discharge (different from the normal) which were not mucoidal discharge (grade 1) were observed after the application of the nanofibers. Additionally, no corneal opacity was found in any of the treated rabbit eyes.

The irritation data given in Table S5 was also considered on the Kay and Calandra (1962) classification of eye irritation scores (see Table 1 in Section 2.3.13). The MMTS score for the blank coaxial fibers was 23.8 (> 15.1 but < 25), while the value was 12.8 (> 2.6 but < 15) for the drug-loaded coaxial nanofibers, as shown in Table 3. Consequently, the blank coaxial fibers were classified as mildly irritating, while the drug-loaded coaxial nanofibers were categorized as minimally irritating. The lower value for the drug loaded systems is surprising and merits further investigation; nevertheless the result is encouraging with regard to the utility of the approach.

Table 3: The MMTS calculations for the blank and drug-loaded coaxial fibers according to the scores from Table S5 in the Supplementary Information section. The drug-loaded and blank coaxial fibers were classified as minimally (12.8) and mildly (23.8) irritating to the rabbit eye tissues, respectively.

Blank coaxial fibers								
Rabbit no.	**1**	**2**	**3**	**4**	**5**	**6**	**SUM**	**Average (SUM/6)**
Cornea	0	20	0	20	0	20	**60**	10.0
Iris	5	10	5	10	5	0	**35**	5.8
Conjunctiva	8	8	8	8	10	6	**48**	8.0
SUM total =	**13**	**38**	**13**	**38**	**15**	**26**	**143**	**23.8**
Drug-loaded coaxial fibers								
Rabbit no.	**1**	**2**	**3**	**4**	**5**	**6**	**SUM**	**Average (SUM/6)**
Cornea	0	0	0	20	0	0	**20**	3.3
Iris	5	5	0	0	5	0	**15**	2.5
Conjunctiva	8	6	6	10	6	6	**42**	7.0
SUM total =	**13**	**11**	**6**	**30**	**11**	**6**	**77**	**12.8**

All rabbits appeared active and healthy with no signs and symptoms of gross toxicity during the course of this irritation study, apart from the reported eye irritation. Additionally, there were no remaining fibers noted in next day eyes' investigation. This might indicate either the complete degradation of the fibers after the 24-hour treatment or the displacement of the fibers from the eye via head movements of the rabbit; no severe irritation was reported that may arise from blocking the lacrimal puncta by the fibers which indicates that tear duct deposition may be unlikely. This minimum irritation was also in agreement with the study by Gagandeep *et al*. (2014) on PVA and PCL nanofibers, as well as the study by Canadas *et al*. (2016) on PLGA nanoparticles.

4. Conclusion

The aim of the investigation was to assess the biological efficacy of the dual drug-loaded nanofibers containing moxifloxacin HCl and pirfenidone, following earlier studies that indicated promising release behavior (Tawfik et al, 2020). This was assessed using three platforms; *in vitro* microbiological assessment, assessment of downregulation of a protein (α-SMA) associated with scarring and *in vivo* studies; involving pharmacokinetic assessment, inhibition of infection and assessment of irritation. After determining minimum inhibitory concentration values against two pathogens (*S. aureus* and *P. aeruginosa*), the nanofibers were shown to preserve the antimicrobial activity of moxifloxacin HCl over a 24-hour period using a zone of inhibition test. In terms of the downregulation of α-SMA, the toxicity against corneal fibroblasts was first assessed in order to allow rational choice of drug exposure followed by a Western Blot assay which indicated comparable α-SMA expression for the nanofibers compared to the free drug over a 24-hour exposure period. On this basis, the antimicrobial and scar prevention properties of dual loaded nanofibers appeared to be largely equivalent to the free solutions of the two drugs, in turn indicating no loss of efficacy following encapsulation. However, the pharmacokinetic profile indicated that the fibers allowed release *in vivo* over a 24-hour period, while the solutions were rapidly eliminated from the cornea. Furthermore, the inhibition of infection for a single dose of the fibers was equivalent to four doses of the moxifloxacin HCl, supporting the suggestion that once daily dosing using the fibers is a viable possibility. There was some evidence for mild ocular irritation when using the fibers which needs to be considered further when refining the delivery system. Nevertheless, the results have indicated that the dual-loaded nanofibers present a viable alternative to repeated solution dosing and that efficacious and practical once daily dosing is possible using the nanofiber approach. Clearly, the use of an *in vivo* scar-inhibition model is required to ascertain the healing propensity of the system, but the results presented here provide an encouraging endorsement of the dosage form at this early stage of development.

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Appendix XVI A. Test for Sterility, BP 2019 (Ph. Eur. 9.6 update) - Appendices, 16 January 2019, <https://www.pharmacopoeia.com/bp-2019/appendices/appendix-16/appendix-xvi-a--test-for-sterility.html?text=test%20for%20sterility&date=2019-01-01>.

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Supplementary Information Section

S.1 Key findings from earlier work (Tawfik et al, 2020)

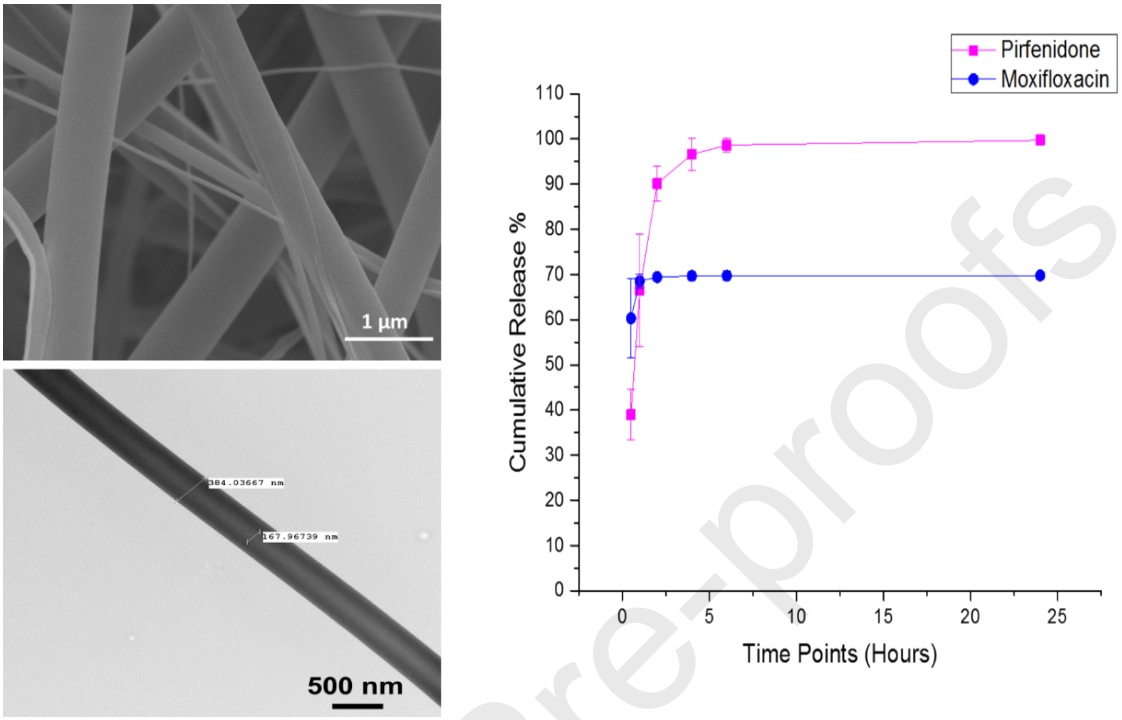


Figure S1: Electron microscopy images (left images) showing the core/shell layers of the drug-loaded coaxial fibers. Release profile (right image) of the drug-loaded coaxial fibers showing a complete release of the pirfenidone after 24 hours, while 68.5% of the moxifloxacin HCl was released over the same time period. Adapted from Tawfik et al, (2020).

S.2 Sterility Testing of the Coaxial Nanofibers

After fabricating the blank and drug-loaded fibers under aseptic conditions, sterility testing was performed according to the British Pharmacopeia (*Appendix XVI A. Test for Sterility,* 2019). As recommended, two media were used to test for both aerobic and anaerobic bacteria, as well as fungi. Both media were incubated and monitored (on a daily basis) for the full sterility test duration (14 days). Figure S2 showed the presence of clear solutions for the tested fibers after 14 days, as well as intentionally contaminated media to confirm the capacities of the media to support microbial growth. For FTG medium, turbidity was the indicator for bacterial growth, while a clear visual fungus growth was the indicator for SBC medium contamination. The lack of turbidity and fungus growth for the blank and drug-loaded fibers after 14 days of incubation was sufficient to confirm the sterility of these fibers and their safety to be used in the following *in vitro* and *in vivo* tests.

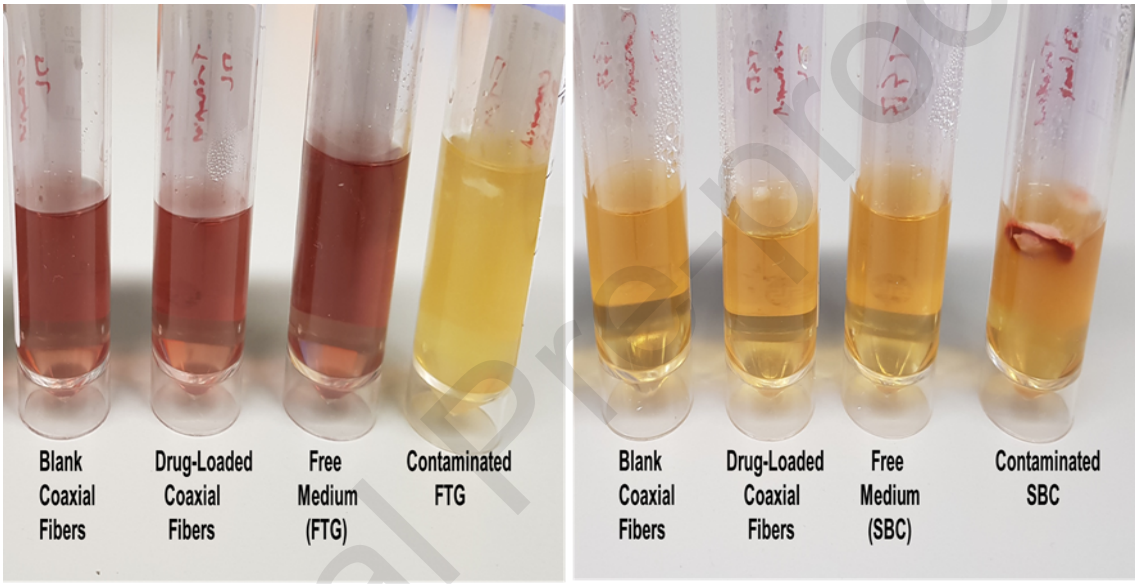
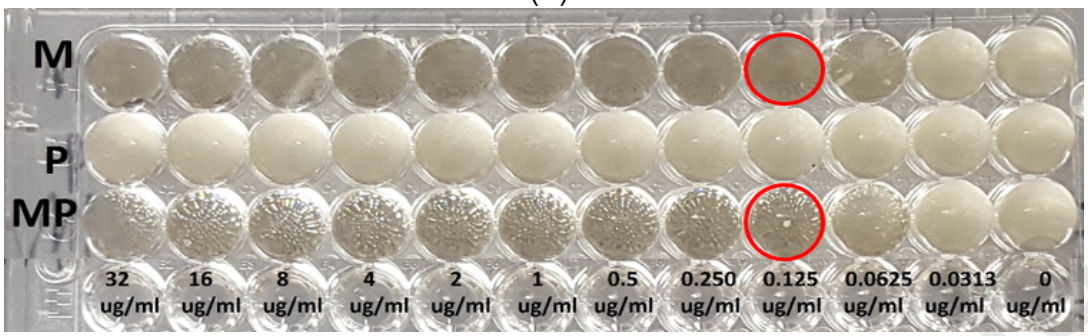


Figure S2: Sterility test after 14 days incubation in FTG medium (left image) and SBC medium (right image). The absence of a change in the medium color or a visible turbidity or fungus growth is an indication for the medium sterility. The FTG contaminated medium (in the left image) showed a change in the medium color and a visible turbidity. The SBC contaminated medium (in the right image) showed a visible growth of a red colored fungus.

S3. Determination of the Minimum Inhibitory Concentration (MIC) of moxifloxacine HCl and pirfenidone against pathogenic bacteria

(A)



(B)

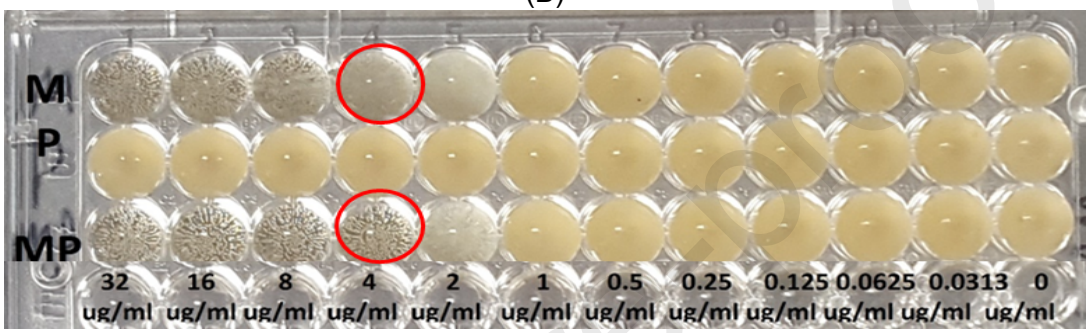


Figure S3: MIC determinations against S. aureus (A) and P. aeruginosa (B). The minimum concentration (red circled) is the lowest concentration where no growth was observed. Moxifloxacin HCl demonstrated an MIC of 0.125 µg/mL and 4 µg/mL against S. aureus and P. aeruginosa, respectively. Pirfenidone demonstrated no activity against either bacterial strains. M: moxifloxacin HCl, P: pirfenidone, MP: moxifloxacin HCl and pirfenidone in 1:1 combination.

S.4 Zone of inhibition studies against pathogenic bacteria

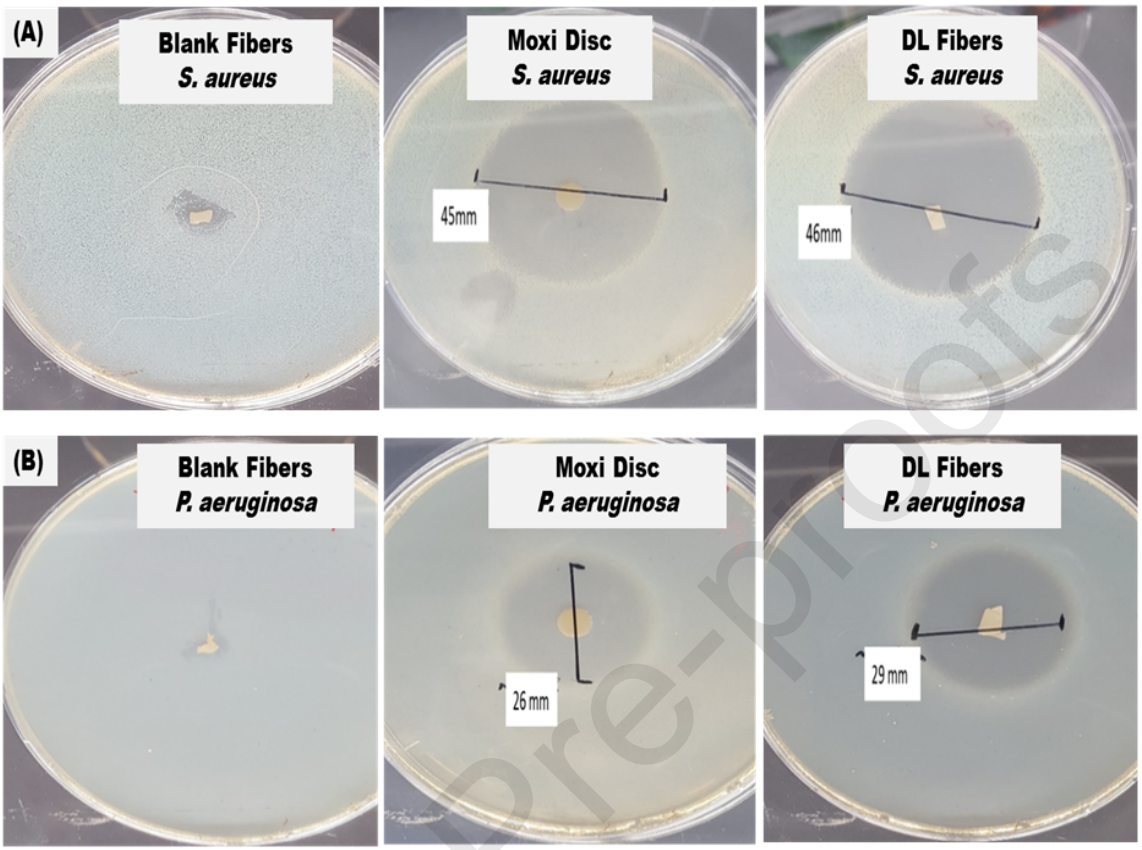


Figure S4: Zone of inhibition against S. aureus (A) and against P. aeruginosa (B). The diameter of the area of no growth is consider as the zone of inhibition. Blank fiber (left), moxifloxacin HCl disc (middle) and drug-loaded coaxial nanofiber (right). The zone of inhibition diameters of the drug-loaded fibers were 45 mm (± 1 mm) against S. aureus and 28 mm (± 1 mm) against P. aeruginosa compared to 42 mm (± 1 mm) and 26 mm (± 1 mm) for moxifloxacin HCl discs, respectively. The blank fibers showed no effect against either bacterial strains.

S.5 Fibroblast compatibility studies

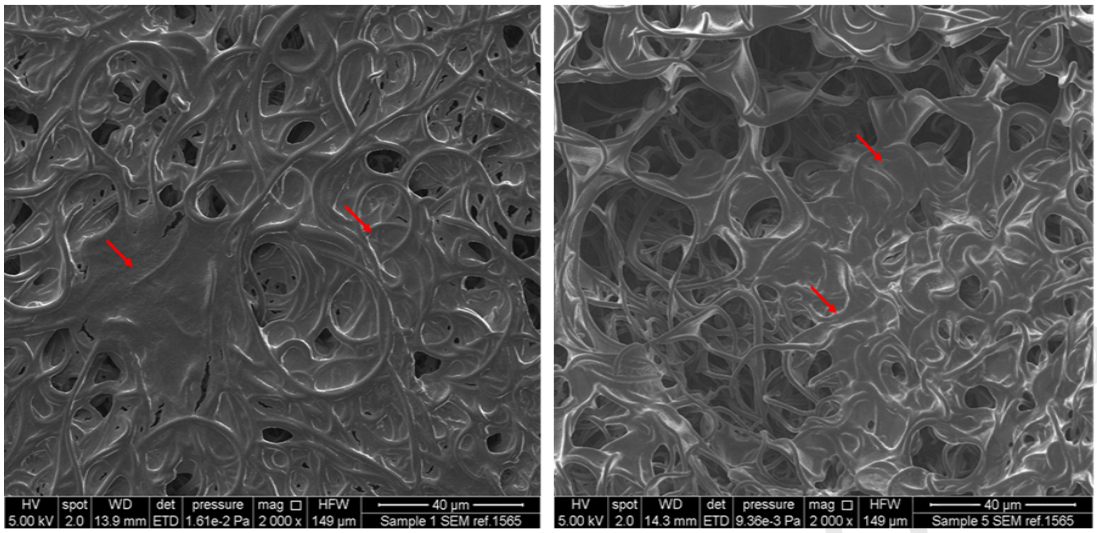


Figure S5: SEM image for cell growth and proliferation on blank (left) and drug-loaded (right) coaxial fibrous membranes. Red arrows indicate the presence of cells growth within the fibrous network.

S6. Determination of Anti-Scarring Proteins using the Western Blot Technique

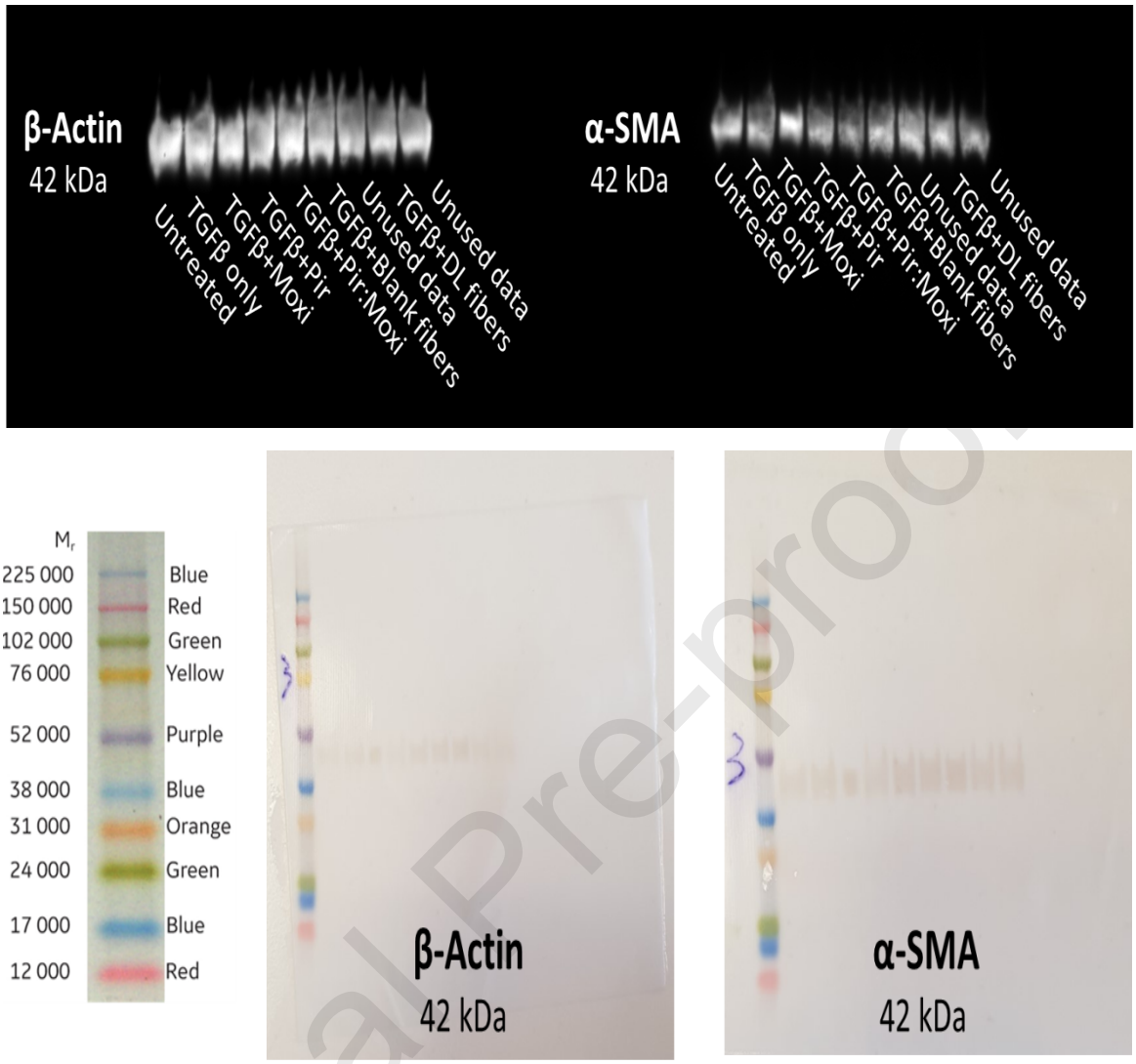


Figure S6: Top image shows the gel electrophoresis of β-Actin (experimental control) and α-SMA (protein of interest), while the bottom image shows the molecular weight range of the Western blot ladder (left); the whole blot of β-actin (middle); right image is the whole blot of α-SMA (right).

S7. UPLC Analysis of moxifloxacine HCl and pirfenidone

For the purpose of analyzing the withdrawn samples from the aqueous humor, a UPLC standard curve was developed. Waters Acquity H-Class UPLC system coupled with a Waters UV detector by Acquity UPLC (Waters, Milford, USA) was used. The UPLC system included a quaternary solvent manager and a sample manager (Acquity UPLC Waters). This assay was developed by combining and modifying the parameters described in Kalam (2016) and the HPLC developed methods described in Tawfik *et al*. (2020). The elution of moxifloxacin HCl and pirfenidone was performed on Acquity UPLC BEH^{TM} C_{18} column (1.7 µm, 2.1 x 50 mm, Waters, USA) maintained at 25°C temperature. An acidic phosphate buffer 20 mM [2.72 g Potassium dihydrogen orthophosphate (MW 136.02) in 1 L of HPLC grade water and adjusted to pH 3.3 by 4-5 drops of phosphoric acid] and ACTN was used as the mobile phase, in a ratio of 65:35, and pumped at a flow rate of 0.12 mL/minute. The injection volume was 5 µL and a total run time of 5 minutes. UV detection was performed at a wavelength of 310 nm.

Standard stock solutions of moxifloxacin HCl and pirfenidone were prepared in methanol to get a final concentration of 50 µg/mL. These stock solutions were diluted with the mobile phase to obtain the working standard solutions of the two drugs in a range of 250-20,000 ng/mL. Working solutions of both drugs were further prepared by taking 20-25 µL aliquots of each working standard solution, where 50 µL of blank rabbit aqueous humor and 130 µL of ACTN were added. The final volume was adjusted to 500 µL by adding 300 µL of mobile phase and vortexed to yield spiked calibration standards (7 points) ranging from 10 to 500 ng/mL.

UPLC Method Validation

This developed UPLC assay was validated according to the International Conference on Harmonisation (ICH) guideline (ICH, 2005) as follows.

a) Specificity

The specificity of this assay is assessed by the separation of both drugs in the presence of endogenous constituents of the aqueous humor. The chromatograms of the different aqueous humor samples obtained through the developed UPLC method are presented in Figure S7. All chromatograms showed no interfering peak in the detection of both drugs, which was consistent with Warsi *et al.* (2012), Sushma *et al*. (2015) and Sun *et al*. (2015). There was a complete separation between moxifloxacin HCl and pirfenidone in the combined samples, with no overlapping peaks. The individual peaks generally showed a good shape, with a degree of asymmetry which was also seen in Warsi *et al*. (2012) Parmar *et al*. (2014) and Dewani *et al*. (2011). The retention times for moxifloxacin HCl and pirfenidone were found to be 1.3 minutes and 2.1 minutes, respectively.

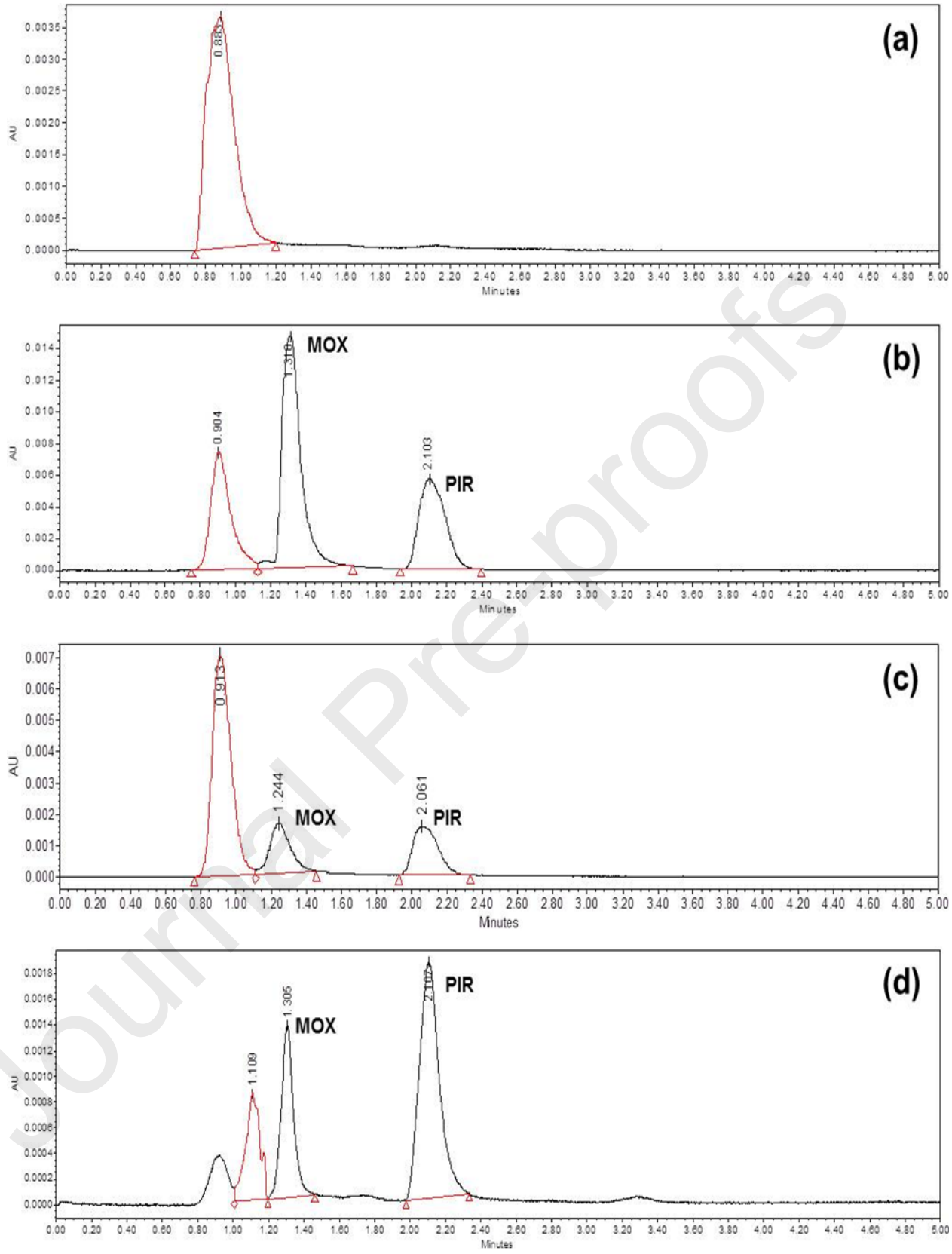


Figure S7: Representative UPLC chromatograms: (a) blank rabbit aqueous humor, (b) aqueous humor spiked with 100 ng/mL of moxifloxacin HCl and pirfenidone solutions, (c) aqueous humor sample 2 hours after topical application of nanofibers and (d) aqueous humor sample 2 hours after topical application of combined drug solution.

b) Linearity and Sensitivity

The linearity of this assay is measured by using 7 concentration points (ranging from 10 to 500 ng/mL). Calibration curves were found to be linear over the concentration range for both moxifloxacin HCl and pirfenidone as shown in Figure S8 and S9, respectively. The calibration curves demonstrated a better linearity for moxifloxacin HCl and pirfenidone with R^2 values of 0.9997 and 0.9913, respectively. This was consistent to Warsi *et al*. (2012), Sushma *et al*. (2015) and Sun *et al*. (2015) who obtained R^2 values above 0.998 for moxifloxacin HCl and 0.994 for pirfenidone which was slightly less than moxifloxacin HCl (Table S1). The sensitivity of the analytical method can be determined by calculating the Limit of Detection (LOD) and Limit of Quantitation (LOQ) using the following equations.

$$LOD = 3.3\left(\frac{SD}{S}\right) \qquad LOQ = 10\left(\frac{SD}{S}\right)$$

Where SD is the standard deviation of the responses, while S is the average value of slope of the calibration curve. The LOD and the LOQ were were found to be 0.045 and 0.137 ng/mL for moxifloxacin HCl and 0.054 and 0.166 ng/mL for pirfenidone. Warsi *et al*. (2012) study demonstrated an LOD and LOQ of 0.04 and 0.12 ng/mL respectively for moxifloxacin HCl detection using UPLC.

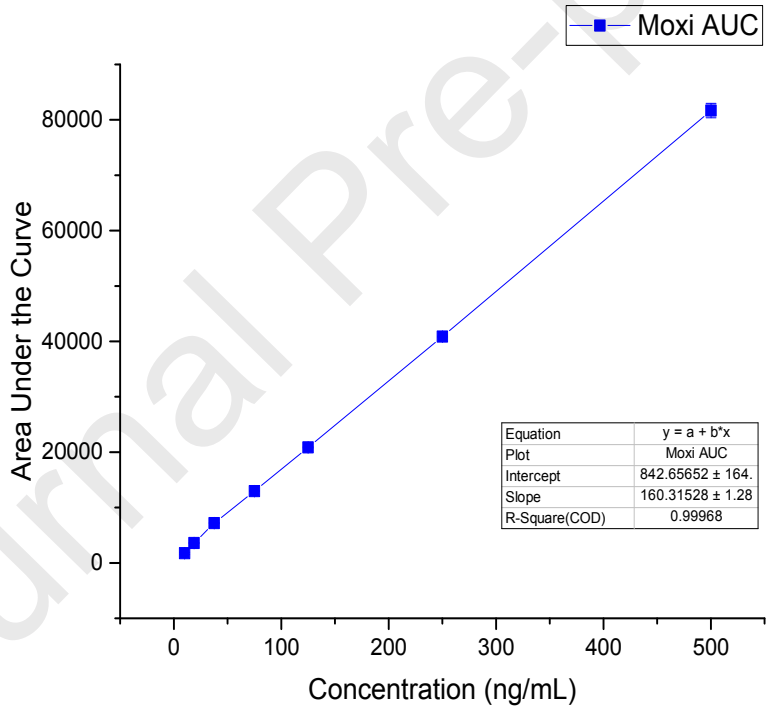


Figure S8: UPLC standard curve of moxifloxacin HCl by plotting the area under the curve against the corresponded concentration. The curve shows good linearity ($R^2 \geq 0.9997$).

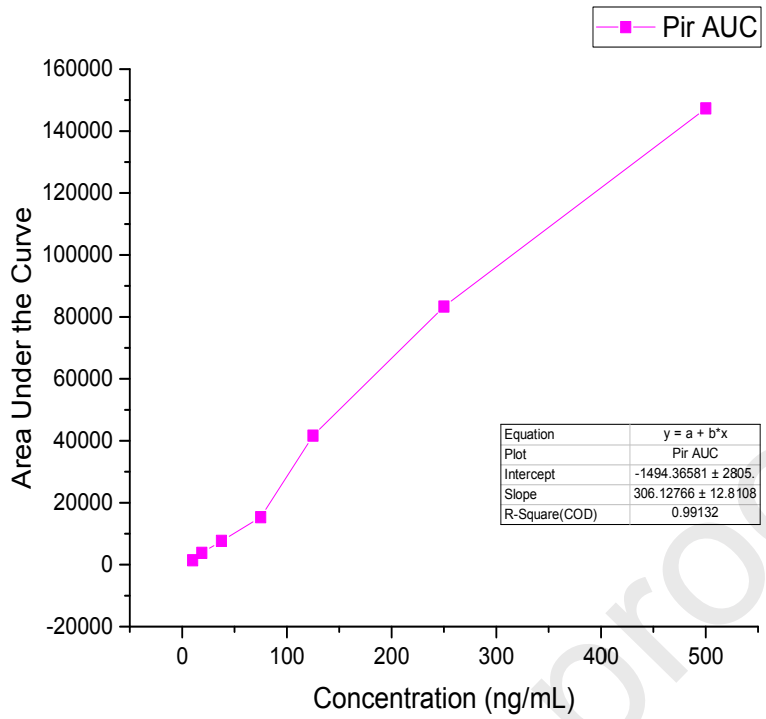


Figure S9: UPLC standard curve of pirfenidone by plotting the area under the curve against the corresponded concentration. The curve shows fair linearity ($R^2 \geq 0.991$).

Table S1: Linear regression data for the calibration curves parameters for moxifloxacin HCl and pirfenidone.

Parameters	Moxifloxacin HCl	Pirfenidone
Linearity range (ng/mL)	10 to 500	10 to 500
Regression equation	Y = 160.32X + 842.66	Y = 306.13X - 1494.37
Correlation coefficient (R^2)	0.9997	0.9913
Retention time (R_t)	1.31 minutes	2.10 minutes

c) Intra- and Inter-Day Precision

Intra- and inter-day precisions can be measured by running each concentration point in triplicate on the same day and in three consecutive days, respectively. Then, the RSD for 3 different concentrations (10, 100, 500 ng/mL) of each drug is calculated. The measured intraday and interday precision values are showed in Table S2. The developed method was found to be precise, as the percentage of the RSD% values for the intraday and interday precisions were below 2.0%, which was consistent with the recommendations of the International Conference on Harmonization guidelines (ICH, 2005), as well as, Parmar *et al*. (2014) and Dewani *et al*. (2011). The percentage recovery values were found in the range of 97.84-99.94% and 98.19-100.02% for moxifloxacin HCl and pirfenidone, respectively, as shown in Table S2. This indicated

the accuracy of the method for the quantification of moxifloxacin and pirfenidone in aqueous humor samples.

Table S2: Intra- and inter-day precision of the developed UPLC method for moxifloxacin HCl and pirfenidone showing the RSD% and recovery% for 3 different concentrations.

Analyte	Nominal Concentration (ng/mL)	Intraday precision		
		Concentration detected (ng/mL) mean ± SD (n=3)	RSD (%)	Recovery (%)
Moxifloxacin HCl	10.00	9.79 ± 0.15	1.52	97.93
	100.00	99.79 ± 0.20	0.20	99.79
	500.00	499.72 ± 0.18	0.04	99.94
Pirfenidone	10.00	9.99 ± 0.19	1.87	99.94
	100.00	99.12 ± 0.75	0.76	99.13
	500.00	499.084 ± 1.86	0.37	99.82
Analyte	Nominal Concentration (ng/mL)	Interday precision		
		Concentration detected (ng/mL) mean ± SD (n=3)	RSD (%)	Recovery (%)
Moxifloxacin HCl	10.00	9.53 ± 0.07	0.75	95.36
	100.00	98.23 ± 0.19	0.20	98.24
	500.00	498.65 ± 0.69	0.14	99.73
Pirfenidone	10.00	10.06 ± 0.19	1.88	100.62
	100.00	99.17 ± 0.86	0.87	99.17
	500.00	499.03 ± 0.97	0.19	99.81

d) Robustness

The intentional modification of the method of analysis, such as a slight change in the mobile pH or flow rate, which may alter the resulting peak was evaluated. The absence of any noticeable change on the chromatogram indicates the recovery of this method of analysis. Therefore, the compositions of the used mobile phase were deliberately changed as ± 2 mL each, pH of the mobile phase (± 0.2) and flow rate of the mobile phase (± 0.2 mL/minute). There was no noticeable effect in the chromatograms and the peak areas, which was consistent with Sushma *et al*. (2015). The values for the robustness of the developed UPLC-UV method are shown in Table S3.

Table S3: Robustness of the UPLC method to analyze 200 ng/mL of moxifloxacin HCl and pirfenidone following changing the mobile phase ratio by ± 2 mL each, pH by ± 0.2 and flow rate by ± 0.2 mL/minute.

Optimized conditions	Obtained conc. for the 200 ng/mL of moxifloxacin HCl		Obtained conc. for the 200 ng/mL of pirfenidone	
	Mean ± SD (n=3)	**RSD (%)**	**Mean ± SD (n=3)**	**RSD (%)**
Mobile phase composition; (20 mM KH_2PO_4 : Acetonitrile; 65:35 ± 2.0)				
(67 : 33, v/v)	199.447 ± 1.974	1.974	199.113 ± 3.820	1.918
(65 : 35, v/v)	200.836 ± 2.431	1.210	198.422 ± 2.074	1.045
(63 : 37, v/v)	199.746 ± 2.186	1.094	199.198 ± 2.174	1.092
Mobile phase flow rate (0.12 ± 0.02)				
(0.14 $mL.min^{-1}$)	199.808 ± 1.092	0.546	199.258 ± 3.673	1.843
(0.12 $mL.min^{-1}$)	199.147 ± 3.031	1.522	200.823 ± 2.566	1.278
(0.10 $mL.min^{-1}$)	199.840 ± 3.222	1.612	198.946 ± 3.408	1.713
pH of 20 mM KH_2PO_4 (3.2 ± 0.2)				
(At pH 3.4)	200.995 ± 1.949	0.969	199.275 ± 2.434	1.221
(At pH 3.2)	200.882 ± 2.474	1.231	200.451 ± 2.621	1.307
(At pH 3.0)	200.729 ± 2.754	1.372	200.486 ± 2.175	1.085

S8. In Vivo Infection Inhibition Study of the Drug-Loaded Coaxial Nanofibers

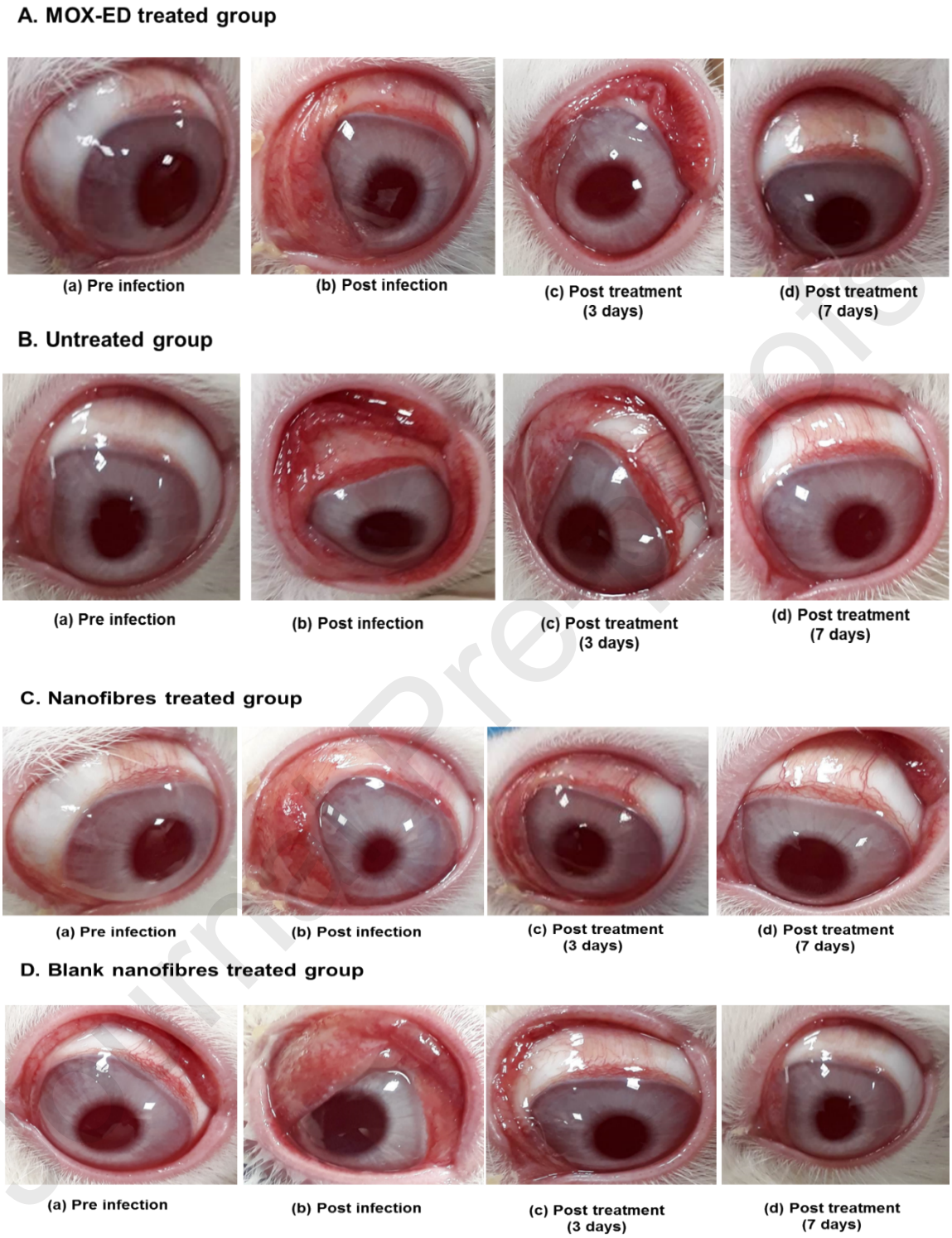


Figure S10: Photographs of the eyes; (a) pre infection (no bacterial growth), (b) 24-hour post infection, (c) 3-day post treatment and (d) 7-day post treatment. A: Moxifloxacin HCl eye drops, B: untreated control group, C: drug-loaded coaxial nanofibers, D: blank coaxial nanofibers.

S9. In Vivo Ocular Irritation Study of the Blank and Drug-Loaded Coaxial Nanofibers

Table S4: Scores for grading the severity of ocular lesions in the ocular irritation studies.

For the cornea	
Lesions	**Score**
i. Opacity-degree of density (area which is most dense is taken for reading)	
Scattered or diffuse area - details of iris clearly visible	**1**
Easily noticeable translucent areas, details of iris slightly obscured	**2**
Opalescent areas, no details of iris visible, size of pupil barely noticeable	**3**
Opaque, iris invisible	**4**
ii. Area of cornea involved	
One quarter (or less) but not zero	**1**
Greater than one quarter but less than one half	**2**
Greater than one half but less than three quarters	**3**
Greater than three quarters up to whole area	**4**
Score equals (i x ii x 5): Total maximum =	**80**
For the iris	
Lesions	**Score**
i. Values	
Folds above normal, congestion, swelling, circumcorneal injection (any one 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 one/ all of these)	**2**
Score equals (i x 5): Total maximum =	**10**
For the conjunctiva	
Lesions	**Score**
i. Redness (refers to palpebral conjunctiva only)	
Vessels definitely injected above normal	**1**
More diffuse, deeper crimson red, individual vessels not easily noticeable	**2**
Diffuse beefy red	**3**
ii. Chemosis	
Any swelling above normal (includes nictitating membrane)	**1**
Obvious swelling with partial eversion of the lids	**2**
Swelling with lids about half closed	**3**
Swelling with lids about half closed to completely closed	**4**
iii. Discharge	
Any amount different from normal (does not include small amount observed in inner canthus of normal animals)	**1**
Discharge with moistening of the lids and hairs just adjacent to the lids	**2**
Discharge with moistening of the lids and considerable area around the eye	**3**
Score equals (i + ii + iii) x 2: Total maximum =	**20**

Table S5: Obtained weighted scores for ocular irritation studies by blank and drug-loaded coaxial nanofibers.

Lesions in the eye structure	Individual scores for ocular irritation											
	Blank nanofibers						Drug-loaded nanofibers					
	Rabbit number						Rabbit number					
	1	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
A. Cornea												
i. Opacity	0	1	0	1	0	1	0	0	0	1	0	0
ii. Area	4	4	4	4	4	4	4	4	4	4	4	4
Total scores obtained = (i x ii x 5) =	**0**	**20**	**0**	**20**	**0**	**20**	**0**	**0**	**0**	**20**	**0**	**0**
B. Iris												
i. Values	1	2	1	2	1	0	1	1	0	0	1	0
Total scores obtained = (i x 5) =	**5**	**10**	**5**	**10**	**5**	**0**	**5**	**5**	**0**	**0**	**5**	**0**
C. Conjunctiva												
i. Redness	1	2	2	1	2	1	2	1	1	2	0	1
ii. Chemosis	2	1	2	2	1	1	1	1	2	2	1	1
iii. Discharge	1	1	0	1	2	1	1	1	0	1	2	1
Total scores obtained =	**8**	**8**	**8**	**8**	**10**	**6**	**8**	**6**	**6**	**10**	**6**	**6**

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Credit author statement:

Author contributions

- ET, DC, AA and SAB were involved in the study design and strategy
- ET performed the majority of the experimental work
- KH assisted and advised on the Western blot assays
- PS assisted and advised on the microbiological studies
- AA, MA-K, MA and MR assisted and advised on the in vivo studies
- All were involved in the preparation of the manuscript

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: