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Surface modification of an intraocular lens material by plasma-assisted grafting with 2-hydroxyethyl methacrylate (HEMA), for controlled release of moxifloxacin

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Abstract

Endophthalmitis, an inflammation of the eye due to perioperative infection, may occur after cataract surgery. Intraocular lenses (IOLs) loaded with an antibiotic have been proposed as an alternative to the conventional postoperative endophthalmitis prophylaxis, since the antibiotic is delivered directly to the target site. In this work, an IOL-based antibiotic releasing system was prepared from a copolymer used in the production of IOLs and a fluoroquinolone used in endophthalmitis prophylaxis (moxifloxacin, MFX). Argon plasma-assisted grafting with 2-hydroxyethyl methacrylate (HEMA) in the presence of MFX was the approach selected for surface modification, with MFX loaded both by entrapment in the grafted polyHEMA coating and by soaking. Surface and bulk properties were evaluated before and after surface modification and the MFX release profiles were obtained both in batch mode (sink conditions) and under hydrodynamic conditions, employing a purpose-built microfluidic cell, which simulated the hydrodynamic conditions around the eye lens. The effect of storage on the release profile of the best system was also assessed. The best system released MFX for ca. 15 days above the minimum inhibitory concentration for *Staphylococcus aureus* and *Staphylococcus epidermidis*. The released MFX showed antimicrobial activity against these bacteria and was non-cytotoxic against corneal endothelial cells.

Keywords

Cataract; endophthalmitis; intraocular lens; controlled release; moxifloxacin; plasma grafting; argon; 2-hydroxyethyl methacrylate

Abbreviations

| | |
|-----------------------|--|
| AFM | Atomic Force Microscopy |
| ca. | <i>circa</i> |
| CEC | Corneal endothelial cells |
| EWC | Equilibrium water content |
| FBS | Fetal bovine serum |
| HBSS | Hank's balanced salt solution |
| HEMA | 2-Hydroxyethyl methacrylate |
| HPLC | High-Performance Liquid Chromatography |
| IOL | Intraocular lenses |
| ISO | International Organization for Standardization |
| K- | Negative control |
| K+ | Positive control |
| m_d | Mass of dry disc |
| MEM | Eagle's Minimum Essential Medium |
| MXF | Moxifloxacin |
| MH | Muller Hinton |
| MIC | Minimum Inhibitory Concentration |
| m_s | Mass of disc at swelling equilibrium |
| MTS | 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt |
| n | Refractive index |
| NADH | Nicotinamide adenine dinucleotide (reduced form) |
| NADPH | Nicotinamide adenine dinucleotide phosphate (reduced form) |
| p | Probability of rejecting the null hypothesis |
| PES | Phenazine ethosulfate |
| PHEMA | PolyHEMA |
| PolyHEMA | Poly(2-hydroxyethyl methacrylate) |
| R_a | Roughness |
| <i>S. aureus</i> | <i>Staphylococcus aureus</i> |
| <i>S. epidermidis</i> | <i>Staphylococcus epidermidis</i> |
| SD | Standard deviation |
| SEM | Scanning Electron Microscopy |
| UV | Ultraviolet |
| Vis | Visible |
| v/v | Volume to volume |
| WCA | Water contact angle |

1. Introduction

Cataracts, the opacification of the eye lens or of the anterior or posterior part of the lens capsule, are the leading cause of visual impairment. In the United States of America (USA), it accounts for 46% of visual disability in people aged 75 to 85 years while, in developing countries such as India, it shows a dramatic rate of 82% among people aged 75 to 83 years [1]. Treatment requires the removal of the natural lens of the eye and implantation of a polymeric intraocular lens (IOL). IOLs are also used in intraocular refractive surgery, where vision correction is addressed employing an IOL with the adequate refractive power. Postoperative endophthalmitis, a purulent inflammation of the aqueous and vitreous humours of the eye, may occur after cataract surgery or after any other type of surgical intervention in the eye [2]. It is usually due to perioperative infection caused by bacteria, fungi or, more rarely, parasites that have entered the eye. It has an incidence up to 0.2% [3] and, if left untreated, irreversible damage to photoreceptor cells of the retina may occur, with partial or complete loss of vision, frequently within a few days [2,4]. Prophylaxis by biocide and antibiotic administration is carried out in every surgical intervention, with protocols which vary with country. In general, preoperative use of povidone-iodine (a topical antiseptic) and preoperative and postoperative topical, subconjunctival or intracameral administration of antibiotics, such as vancomycin, cefuroxime and fluoroquinolones, are employed [5]. In Europe, a survey which involved nine countries revealed that, in general, preoperative antisepsis employing topical application of povidone-iodine or chlorhexidine, associated with postoperative topical antibiotic prophylaxis for up to 2 weeks, or intracameral cefuroxime injection at the end of surgery, are the most common approaches [6]. Another survey found that most European surgeons now favour intracameral administration of antibiotics over subconjunctival administration or topical antibiotics alone [7]. In the USA, a different approach is taken: the most common form of prophylaxis has been the use of topical fluoroquinolones prescribed 1 to 3 days preoperatively and resumed immediately postoperatively for 1 week [3,8]. Intracameral administration has the advantage of providing far higher antibiotic concentrations at the target site than subconjunctival injection or topical administration (eye drops) [7]. However, it is an invasive procedure. Eye drops are a simple ocular drug administration method but suffers from: (i) low bioavailability, due to anatomical and physiological barriers (blinking, lacrimation, flow through the nasolacrimal duct, rapid absorption

into the bloodstream and poor corneal penetration), and (ii) poor compliance, due to inadequate postoperative use and timing by patients who, in most cases, show some age-related physical impairment. As such, less than 5% of the administered drug enters the eye [9], and the required therapeutic concentration may not be attained.

IOLs loaded with an antibiotic have been proposed as an alternative to both intracameral injection and eye drops in endophthalmitis prophylaxis, since drug delivery directly to the target site is achieved. In the 90's, Nishi *et al.* [10] and Tetz *et al.* [11] were among the pioneers who proposed and showed the efficacy of IOLs as drug delivery systems, obtaining reduced eye inflammation and reduced posterior chamber opacification (a complication of cataract surgery), employing IOLs loaded with anti-inflammatory and anti-proliferative drugs. In the following decade, Shimizu *et al.* [12] and Kleinmann *et al.* [13] were among the first to propose the use of antibiotic-loaded IOLs for endophthalmitis prophylaxis. In the first study, it was shown that polymethacrylate IOLs loaded with fluoroquinolones had a preventive effect against *in vivo* bacterial biofilm formation (employing a rabbit endophthalmitis model) [12] and, in the second study, which employed a fluoroquinolone (moxifloxacin; MFX) and polymethacrylate IOLs, *in vivo* concentrations above the Minimum Inhibitory Concentration (MIC) for bacteria associated with endophthalmitis were sustained for 14 to 16 h by drug release from IOLs soaked for 24 h in a MFX solution at 5 mg/mL [13]. A study with the rabbit model, which compared the use of an antibiotic-loaded hydrophilic acrylic IOL to intracameral antibiotic injection for endophthalmitis prophylaxis with fluoroquinolones, showed that the effect against bacterial proliferation was similar for both drug administration types [14].

IOLs have been loaded with different types of drugs, including antibiotics [15], anti-inflammatory [10] and anti-proliferative drugs [11]. The drugs were located (i) in the IOL itself, (ii) in a coating applied onto the IOL or (iii) in a separate reservoir attached to the IOL [16]. However, in spite of more than 20 years of research, drug-loaded IOLs have not become commercially available and currently, there are no clinical trials in the european [17], american [18] or japanese [19-21] clinical trials registers.

The objective of this work was to develop antibiotic releasing systems for use in prophylaxis of postoperative endophthalmitis, employing discs made from a hydrophilic, polymethacrylate-based copolymer used in the production of IOLs. These systems aim to

release an antibiotic at a concentration above its MIC for bacteria commonly associated with endophthalmitis, such as *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* (*S. epidermidis*) [2], during the required period of time (10 days, at least [3,5,22]). Moxifloxacin (MFX), a fourth generation fluoroquinolone used in endophthalmitis prophylaxis [23], was the antibiotic selected, due to its potency and use in the context of endophthalmitis prophylaxis [24,25], its antifungal activity [26], and its good photochemical [27] and thermal [28] resistance. This last characteristic is of utmost importance, since autoclaving is the method employed industrially to sterilize IOLs prepared with the material used in this work. Approaches based on surface modification by coating were adopted, since thin coatings (i) do not compromise bulk IOL properties, (ii) can act as a barrier to the release of the drug loaded in the IOL, and (iii) can be loaded with extra drug by entrapment. Surface modification of IOLs started probably in the 1970s, when dip coating of polymethacrylate IOLs with polyvinylpyrrolidone [29] and both plasma-assisted and gamma radiation-assisted grafting with 2-hydroxyethyl methacrylate (HEMA) and vinylpyrrolidone [30] were employed as a means to create lubricious coatings in the context of prevention of corneal endothelial damage. In this work, the approach selected for surface modification was argon plasma-assisted grafting with HEMA, with MFX loaded both by entrapment in the grafted polyHEMA (PHEMA) coating and by soaking. PHEMA, a hydrophilic polymer, was selected, since hydrophilic IOLs tend to show lower incidence of both bacterial adhesion and endophthalmitis than hydrophobic IOLs [31]. Additionally, HEMA is a monomer adequate for use in IOLs, since it is one of the monomers present in the formulation of the material used in this work. Plasma-assisted grafting copolymerization presents the following advantages: (i) plasma only penetrates a few nanometers beyond the surface of samples [32], keeping the bulk of the sample unchanged, (ii) surface modification by grafting can be achieved without the use of polymerization initiators, which may be difficult to extract from the modified IOL, and (iii) plasma technology is already used industrially for surface modification of ophthalmic lenses, such as contact lenses and IOLs [33,34]. We used low-pressure plasma as a means to create reactive species on the material's surface (in particular, free radicals, anions and cations), which can initiate graft copolymerization from the surface after contact between the plasma-activated surface and a vinyl-type monomer in the liquid phase.

The surface and bulk properties of the material were evaluated before and after surface

modification, namely wettability, equilibrium water content (EWC), topography/morphology, transmittance, refractive index and coating thickness. The release profiles of MFX-loaded discs were obtained both in batch mode (sink conditions, i.e., employing a volume of release medium that was at least 3 to 10 times the saturation volume) and in a microfluidic cell which simulated the hydrodynamic conditions around the eye lens. The effect of storage on the release profile of the best system was assessed, as well as the best system's antibacterial activity against relevant bacteria (*S. aureus* and *S. epidermidis*) and its cytotoxicity towards relevant cells (rabbit corneal endothelial cells).

2. Materials and Methods

2.1. Materials

Discs made from a hydrophilic poly[(2-hydroxyethyl methacrylate)-co-(methyl methacrylate)]-based material (EWC: 26%) used in the production of IOLs were provided by PhysIOL S.A. (Liège, Belgium). They contained a proprietary yellow chromophore which acts as a blue light filter [35]. The discs, which had a diameter of 1.6 cm and a thickness of 1 mm, were Soxhlet-extracted with distilled water (ca. 60 extraction cycles) before use, and were cut to the required diameter with a cork borer. 2-Hydroxyethyl methacrylate (HEMA) was supplied by Sigma-Aldrich (St. Louis, USA) and moxifloxacin hydrochloride (MFX) was supplied by TSZCHEM/BioTang (Lexington, USA). Hank's balanced salt solution (HBSS) was prepared by dissolving 8 g/L of NaCl, 0.4 g/L of KCl, 0.0356 g/L of Na₂HPO₄, 0.06 g/L of KH₂PO₄, 0.144 g/L of CaCl₂, 0.12 g/L of MgSO₄ and 0.35 g/L of NaHCO₃ in Milli Q water, and adjusting the pH value to 7.4. Culture medium for the antimicrobial activity evaluation (Mueller-Hinton agar) and blank antimicrobial susceptibility testing discs were from Oxoid (Basingstoke, UK). For the cytotoxicity evaluation, the determination of the number of viable cells was carried out with the MTS proliferation assay, employing the CellTiter 96[®] AQueous One Solution Reagent from Promega (Madison, USA), which contains MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] and PES (phenazine ethosulfate). Fetal bovine serum (FBS) was purchased from Biochrom AG (Berlin, Germany) and Eagle's Minimum Essential Medium (MEM), amphotericin B and trypsin were purchased

from Sigma-Aldrich (St. Louis, USA). All chemicals and reagents were of analytical grade and were used as supplied.

2.2. Plasma surface modification

Surface modification by plasma only or by plasma-assisted grafting with HEMA was conducted in a commercial low-pressure plasma reactor (FEMTO, Diener Electronic GmbH, Ebhausen, Germany), composed of a stainless steel chamber of 100 mm diameter and 270 mm length. Discs (diameter: 5 mm) were placed at 80 mm from the electrode and treated as follows: 1) Treatment with argon or oxygen plasma only: samples were exposed to argon or oxygen plasma generated at a pressure chamber of 0.6 mbar, for 1 to 4 min, applying a power of 100 W; 2) Ar plasma-assisted grafting with HEMA: samples were exposed to argon plasma as in 1) and then were immediately immersed in a 10% (v/v) solution of HEMA in HBSS containing MFX at 5 mg/mL, and kept for 1 h at 60 °C. Finally, all modified samples were thoroughly washed with distilled water.

2.3. Drug loading

MFX was loaded into the discs (diameter: 5 mm; dry weight range: 12.1 to 17.6 mg) in two different ways (Scheme 1): 1) Soaking, through immersion of the sample in 250 μ L of a 5 mg/mL MFX solution in HBSS for 24 or 72 h, at 4 °C, without shaking, or for 15 h at 37 °C, under shaking at 100 rpm; 2) Entrapment in the grafted PHEMA coating, by carrying out the grafting with a 10% (v/v) HEMA solution in HBSS which contained MFX at a concentration of 5 mg/mL. After modification, samples were quickly rinsed with distilled water, the excess water was blotted from the surface and, finally, discs were vacuum dried at room temperature before use. Scheme 1 indicates the different types of unmodified and modified, MFX-loaded samples employed in this study (discs #1 to #4).

In the studies where the effect of storage in an MFX solution was evaluated, triplicate discs were stored in a 5 mg/mL MFX solution in HBSS, in a closed vial, at room temperature, for periods ranging from 24 h to 3 months. All drug loading/release studies were performed in triplicate.

2.4. Drug release studies

The drug release studies were carried out by placing the MFX-loaded samples individually in a closed vial containing 3 mL of HBSS at 37 °C, under constant shaking at 100

rpm. At predetermined time intervals, 0.5 mL of the release medium was sampled and replaced with the same volume of fresh HBSS, to imitate the drainage of the aqueous humor and to maintain sink conditions. The MFX concentration present in the collected samples was determined by UV-vis spectrophotometry at 290 nm, employing a Jasco V550 UV-vis spectrophotometer (Jasco Corp., Japan). Blank samples (unmodified, unloaded discs) were also studied. For each time point, the residual absorbance of the blanks was subtracted from the absorbance of the sample under study. The difference in the absorbance between the loaded and the blank samples was used to calculate the drug concentration in the release media and the cumulative amount of drug released per mass of dry disc. Each data point was the average of results from three samples. Results were expressed as mean \pm standard deviation (SD).

The release study was also performed under dynamic conditions, employing a purpose-built microfluidic cell (Figure 1) to simulate the hydrodynamic conditions of the anterior chamber of the eye, which was based on another microfluidic cell developed by us for contact lenses [36]. The cell was conceived in poly(methyl methacrylate) with a cylindrical inner chamber of 250 μ L capacity, identical to the average volume of the aqueous humor present in the eye [37]. It accepted discs with a diameter of 5 mm. The HBSS solution was admitted by 8 radial entry points in the inner chamber of the cell and collected from a central exit point. In order to approximate the *in vitro* experimental conditions to the physiological conditions, the experiments were performed at 37 °C with a continuous HBSS flow of 2.5 μ L/min, respecting the aqueous humor renovation rate of \sim 1%/min [37]. The out flow solution was collected at predetermined time intervals and analyzed for drug quantification by high-performance liquid chromatography (HPLC), employing a Waters e2695 chromatograph with a Nova-Pak C-18 column (both from Waters Corp., Milford, USA) and a Jasco UV-vis detector (Jasco Corp., Tokyo, Japan) set at 290 nm. The chosen mobile phase, methanol and phosphate buffer (pH 3, 55/45, v/v), was run through the column at a flow rate of 1 mL/min and at a pressure of 14 MPa.

2.5. Materials characterization

2.5.1. Wettability

Wettability of both dry and hydrated discs was evaluated by contact angle goniometry. When the effect of the plasma exposure time on the samples was studied, the measurement of

the water contact angle was performed approximately 5 min after sample exposure to plasma. For dry samples, the water contact angle of, at least five, 2 μ L sessile water drops per sample was measured with an OCA 20 goniometer (Dataphysics, Filderstadt, Germany) immediately after settling on the surface, by fitting the Young-Laplace equation to the drop profile. In the case of hydrated samples, the captive air bubble method was employed, using a measuring cell containing water. Images of bubbles attached to the immersed surface were acquired by a video camera (JAI CV-A50, JAI Ltd., Yokohama, Japan) mounted on an optical microscope (Wild M3Z, Wild-Heerbrugg AG, Heerbrugg, Switzerland) and connected to a frame grabber (DT3155, Data Translation, Inc., Marlboro, USA). The acquisition and analysis of the images was performed using the ADSA-P software (Axisymmetric Drop Shape Analysis-Profile, [38]). Ten bubbles were measured per sample. In all cases, results were presented as the average contact angle \pm SD.

2.5.2. Equilibrium water content

The EWC was determined by immersing pre-weighted vacuum-dry discs in the drug loading/release medium at room temperature, until the weight of the swollen discs reached equilibrium. It was defined as the percentage of water in the swollen gel and calculated according to Equation 1, where m_s is the mass of the disc at swelling equilibrium and m_d is the

$$\text{EWC (\%)} = 100 \times (m_s - m_d)/m_s \quad (\text{Equation 1})$$

mass of the dry disc. Assays were carried out in triplicate and results were expressed as mean \pm SD.

2.5.3. Morphology and topography

Scanning Electron Microscopy (SEM) was used to visualize and study sample cross-sections. Samples were fractured after immersion in liquid nitrogen, cross-sections were mounted on stubs and sputter coated with a gold layer (thickness: 10-15 nm) using an Edwards EXC sputter coater (Edwards Vacuum, Crawley, UK) with a Huttinger PFG 1500 DC power supply (TRUMPF Hüttinger GmbH, Freiburg, Germany). Images were acquired with a Field Emission SEM (Zeiss Merlin Compact VP FE-SEM, Carl Zeiss AG, Jena, Germany), employing an accelerating voltage of 10 kV.

Topographic images of the hydrated samples were obtained using an Atomic Force Microscope (AFM) Nanosurf EasyScan 2 (Nanosurf AG, Liestal, Switzerland). The analyses

were done in contact mode, at room temperature, using silicon probes (force constant 0.01–1.87 N/m), with an applied force of 20 nN and at a scan rate of 1.4 Hz. The average roughness (R_a) of the surfaces was obtained considering the total area of the images (20 μm \times 20 μm).

2.5.4. Coating thickness and refractive index

The thickness of the coating layer on the modified discs and the refractive index of both unmodified and modified discs, were determined by ellipsometry (HORIBA UVISEL spectroscopic ellipsometer, from HORIBA Ltd., Kyoto, Japan), through measurement of the relative changes in amplitude and phase of the polarized incident light before and after reflection on the surface of the samples, employing an incidence angle of 70°, in the 300–850 nm wavelength range. Modeling was done with the Deltapsi software associated with the equipment. The experimental data were modeled with a Cauchy transparent model, to extract the optical constants and the layer thicknesses. The tests were done in triplicate using hydrated samples, after careful removal of the excess of water from the surface.

2.5.5. Transmittance

The transmittance of visible light through the hydrated samples was determined using a ThermoScientific Multiscan GO UV–vis spectrophotometer (Thermo Fischer Scientific, Inc., Waltham, USA). Measurements were performed in the 400 to 700 nm wavelength range. Fully hydrated samples were mounted on the inner frontal face of a quartz cuvette. The tests were done in triplicate.

2.6. Antibacterial activity evaluation

Microbiological tests were carried out to assess the efficacy of the drug loaded, modified discs against *S. aureus* and *S. epidermidis*. Two types of evaluations were performed:

1) Evaluation of the activity of MFX released by the loaded discs during the studies in the microfluidic cell, and 2) Evaluation of the activity of MFX-loaded discs by direct contact. Prior to microbiological tests, *S. aureus* ATCC 25923 and *S. epidermidis* CECT 231 were incubated at 37 °C for 24 h. The growth was suspended in a 0.9% NaCl sterile solution to give an optical density of 1 McFarland. Mueller-Hinton (MH) culture medium was prepared according to the producer recommendations and sterilized at 121 °C, during 20 minutes. After MH medium temperature stabilization, 350 μL of bacterial suspensions were added to 50 mL of medium, carefully homogenized and placed in square Petri dishes (120 mm \times 120 mm). After gelification,

blank filter paper discs were distributed on the surface of the agar in the dishes. Solutions collected at days 3, 4 and 7 of the microfluidic cell release experiment were selected for evaluation and 15 μ L of each solution were placed on the paper discs for determination of the antibacterial activity of the antibiotic contained in the solutions, through the *in vitro* diffusion method. For the direct contact assay, drug-loaded discs were directly placed in the plates with the bacteria. The plates were then kept at 37 °C and, after 24 h, the diameters of the inhibition halos were measured with an electronic caliper. The MIC of MFX for both bacteria was determined with solutions of known concentration (between 0.25 and 16 μ g/mL) following a procedure similar to that described for the collected solutions. For each bacterium, a calibration curve that relates the diameter of the inhibition halos with the concentration of drug in drug solutions prepared, was employed.

2.7. Cytotoxicity evaluation

Cytotoxicity evaluation was performed according to the ISO 10993-5:2009 guidelines [39]. Rabbit corneal endothelial cells (CEC) were obtained as previously reported [40]. To assess cell adhesion and proliferation in the presence of the discs, samples with a diameter of 2 mm (quadruplicates) were placed in a 96-well plate and sterilized by UV radiation for, at least, 30 minutes. CECs were then seeded at a density of 2×10^4 cells/well in 200 μ L of MEM, and incubated at 37 °C in a humidified, 5% CO₂ atmosphere. Cell growth and morphology was monitored using an Olympus CX41 inverted light microscope (Olympus, Tokyo, Japan) equipped with an Olympus SP-500 UZ digital camera, at 1, 2 and 5 days.

To characterize cell viability in the presence of discs, CECs were seeded in 96-well plates containing the discs and MEM, and incubated as referred above. After the selected incubation periods (1, 2 and 5 days), cell viability was evaluated using the MTS assay by replacing the culture medium by 100 μ L of fresh medium and 20 μ L of MTS. After an incubation period of 4 h at 37 °C in a humidified, 5% CO₂ atmosphere, the absorbance at 492 nm of each well was determined with a microplate reader (Bio-Rad xMark microplate spectrophotometer; Bio-Rad Laboratories, Inc, Hercules, USA). Wells containing cells in the culture medium without discs were used as negative control (K-). The positive control (K+) was obtained by adding ethanol at 96% to wells containing cells without discs. Cell viability was expressed as a percentage of the negative control.

2.8. Statistical analysis

Comparisons between two means were carried out employing a two-tailed, unpaired Student's *t* test. For comparisons between more than two means, a one-way ANOVA was employed, followed by Dunnett's post test for multicomparisons between each mean and a control. All statistical analyses were carried out at a confidence level of 95%.

3. Results and Discussion

3.1. Plasma exposure conditions

The surface modification method selected for modification of the IOLs employed in this work was Ar plasma-assisted PHEMA-grafting from the surface. In this approach, the material was exposed to Ar plasma with the aim of creating reactive species on its surface (free radicals, anions and cations), which are able to initiate chain-reaction polymerization of a vinyl monomer. After exposure to plasma, the plasma-activated surface was immediately immersed in a solution of a vinyl monomer (HEMA) to achieve PHEMA grafted onto the surface (grafting from the surface). As polymer surfaces exposed to plasma become more hydrophilic, due to formation of oxygen-containing groups on the surface and/or removal of adsorbed contaminants [41,42], we verified which plasma conditions would increase the surface hydrophilicity most significantly, by varying the plasma exposure time and the gas type. Wettability was used as an indicator of surface hydrophilicity and was assessed by measuring the water sessile contact angle on the surface. Two gas types were employed: a reactive gas (oxygen) and an inert gas (argon). As expected, plasma treatment with both gas types decreased the water contact angle when compared to the unmodified surface, implying that surface hydrophilicity increased (Figure 2). As hydrophilicity was higher for an exposure of 3 min to Ar plasma, these conditions were selected for the subsequent HEMA grafting studies.

3.2. Characterization of the surface modification

Contact angle goniometry and water content

Contact angle goniometry was used to compare the wettability of the surfaces of unmodified discs and of discs modified by Ar plasma-assisted grafting with HEMA. When employing dry discs, a statistically significant decrease was detected on the contact angle (sessile drop) upon modification (Table 1). On the contrary, the sessile contact angle on hydrated samples (captive

bubble) did not change significantly after modification. These different results obtained with dry and with hydrated samples may be attributed to reorientation of the grafted PHEMA chains according to the surrounding environment, i.e., exposure of nonpolar groups when placed in air (dry samples) and exposure of polar groups when in contact with water (hydrated samples) [43].

The EWC of the discs was not altered by the surface modification (Table 1), implying that the grafted PHEMA layer did not alter the swelling capacity of the discs. This is in accordance with the low thickness of the grafted layer (~ 52 nm, Table 1).

Ellipsometry

Surface characterization by ellipsometry confirmed the existence of a coating on the modified samples, with a thickness of 52 ± 5 nm (Table 1). The refractive index of the modified samples was not significantly different from that of unmodified samples (Table 1), indicating that the refractive index of the material was not altered by the modification.

Microscopy: SEM and AFM

Cross-sections of samples were observed by SEM, with the aim of visualizing the PHEMA coating. A continuous coating layer could be seen on the modified disc (Figure 3B), which was not present in the unmodified disc (Figure 3A). The thickness of this coating was *ca.* 73 nm, a value close to that calculated from the ellipsometry results (Table 1).

AFM images of the surface of unmodified discs showed a pattern of parallel grooves and an average roughness of 12 ± 2 nm (Figure 4A; Table 1). This pattern is attributed to machining during the disc manufacturing process. After the modification, the grooves were no longer visible and surface roughness increased to 34 ± 2 nm, due to the presence of several protuberances. From the AFM image analysis, it could be inferred that a coating with a thickness above 45 nm would be required to hide this surface pattern. This value is in agreement with the coating thickness obtained by ellipsometry (Table 1). The protuberances seen on Figs. 4A and 4B can be attributed to surface debris and/or to zones where high grafting of PHEMA occurred.

Transmittance

The transmittance spectra of the unmodified discs and of the discs modified by Ar plasma-assisted grafting with HEMA can be seen in Figure 5. In both cases, the transmittance below 500 nm was quite low, due to the blue light filter present in the discs. The filter absorbs a large

part of the high-energy, visible blue light between approximately 380 and 500 nm and also filters ultraviolet light. Blue light filtering IOLs were introduced in cataract surgery in the 1990s, due to evidence of improvements in clarity of vision, contrast acuity, and reduction in glare [44]. The presence of the coating affected only slightly the transmittance of the samples. The average transmittance in the 500-700 nm wavelength range decreased from $94 \pm 2\%$ to $91 \pm 2\%$ after modification.

3.3. Drug release studies

MFX was loaded into the discs in two different ways: (i) by soaking, i.e., by immersion of the discs in a 5 mg/mL MFX solution in HBSS, under different conditions; and (ii) by entrapment of MFX in the grafted PHEMA layer, through grafting with a monomer solution which contained MFX also at a concentration of 5 mg/mL. The loading conditions (soaking time, temperature and absence/presence of shaking) were varied with the aim of increasing the amount of released drug and decreasing the loading time. Four different types of MFX-loaded discs were studied (Scheme 1).

Soaking of discs in a 5 mg/mL MFX solution in HBSS did not allow the quantification of loaded drug by depletion, since the absorbance of the MFX solution before and after the loading was almost the same. The release curves of unmodified discs loaded with MFX by soaking in a 5 mg/mL MFX solution in HBSS for 72 h, at 4 °C and without shaking showed that $\sim 2.5 \mu\text{g}$ of MFX/mg of dry disc was released when the plateau was attained, and that most of the loaded drug was released within the first 24 hours (Figure 6A; discs #1). This duration release was insufficient for the intended application (endophthalmitis prophylaxis), which requires a release duration of, at least, 10 days [3,5,22]. In order to delay the release of the drug, a PHEMA coating was created by Ar plasma-assisted grafting with HEMA. Additionally, extra drug was loaded in the coating by entrapment, by having MFX dissolved in the HEMA solution.

To avoid exposure of the drug to the plasma and drug loss during the washing steps, the modification of the discs was started with discs not loaded with MFX. The drug was loaded by soaking only after the plasma-assisted grafting step, under the same conditions of the soaking of unmodified discs, albeit a much longer time was selected (72 h; discs #3). The modified and loaded discs (discs #3, Figure 6A) released more drug than the unmodified discs (discs #1, Figure 6A), reaching a value of $\sim 5 \mu\text{g}$ of MFX/mg of disc. The release duration was

also impressively increased to ~10 days, a release time which is sufficient for the intended application.

As the drug-loading step had an inconvenient large duration (72 h), and as MFX is thermally stable [28], we tried to shorten the loading time to 15 h (overnight) by increasing the loading temperature to 37 °C and by employing shaking. For unmodified discs, this change allowed an increase of the released MFX from ~2.5 to ~4 µg of MFX per mg of dry disc (Figure 6A, discs #1 and #2, respectively). When these loading conditions were employed in the drug-loading step of the modified discs, the release curve obtained (Figure 6A; discs #4) showed that more drug could be released than when the loading step of modified discs was carried out for 72 h at 4 °C without shaking (Figure 6A; discs #3), increasing from ~5 to ~6.5 µg of MFX/mg of dry disc, respectively, while keeping the same release duration. Thus, a shorter soaking time under shaking and at a higher temperature, allowed a release of the largest amount of drug for, at least, 10 days.

A direct comparison of our release results in batch mode with *in vitro* literature results is not possible, since the very few published *in vitro* studies in which MFX release from MFX-loaded IOLs was studied in batch mode employed different drug soaking times and temperatures, and IOLs were used instead of discs [45]. However, the released MFX concentrations of our best system (discs #4, Fig. 6A) were (i) higher than in a study in which IOLs were loaded by soaking in Vigamox (MFX-containing eye drops, at a concentration of 5 mg/mL) for 10 min (0.7 vs. 0.3 µg/mL of MFX, respectively, after 30 min of release, the only release time studied in the literature study [46]), although our soaking time was much longer and at a higher temperature, and (ii) lower than in a study in which IOLs were soaked in Vigamox for 24 h (20 µg/mL vs. 75 µg/mL, respectively, after 24 h, the longest release time of the literature study [15]), although our soaking time was shorter (15 h) and at a higher temperature (37 °C).

Although the MFX-loaded, modified discs seem to be promising for endophthalmitis prophylaxis, as far as the drug release duration is concerned, as these studies were carried out in batch mode, under sink conditions, it is not clear if the drug release duration would be similar under the hydrodynamic conditions of the aqueous humour in the eye. Additionally, it was not clear for how long the concentration of MFX released under hydrodynamic conditions was

above the MIC for bacteria commonly associated with endophthalmitis, such as *S. aureus* and *S. epidermidis*, which has a value of 0.25 µg/mL for these species (section 3.4). To be closer to the *in vivo* situation, a microfluidic flow cell was employed. It had a chamber volume equivalent to the normal aqueous humor volume (250 µL), in which the aqueous medium (HBSS) was renovated at a flow rate similar to the normal renovation flow rate of aqueous humor (2.5 µL/min) [37]. The evolution of the concentration of the released MFX with time for the most promising system ((Figure 6B, discs #4) showed that this system sustained the release of MFX at a concentration above the MIC for *S. aureus* and for *S. epidermidis* for *ca.* 12 days.

A direct comparison of this result with literature results is not possible, since most equivalent literature results are from *in vivo* studies with IOLs, in which the MFX concentration was determined in the aqueous humor of the anterior chamber of the rabbit eye, while ours are *in vitro* studies employing discs, albeit of a comparable diameter (5 mm). However, our release duration of 12 days above the MIC for relevant pathogens (discs #4) is much longer than in the *in vivo* literature studies with MFX-loaded IOLs [13, 15, 46-50], although the literature studies were not concerned with long-term but with short-term MFX release (maximum release time studied: 24 h). Thus, the modification and loading approach employed in discs #4 resulted in a system releasing MFX at a concentration and with a duration which seems suitable for postoperative endophthalmitis prophylaxis.

For drug-loaded IOLs to be used by surgeons during cataract or refractive surgery, they must reach them in a form which is ready for immediate use. This requires storage of hydrated, MFX-loaded IOLs. For this to be possible without affecting the drug content of the IOL, storage has to be carried out in an MFX solution with a concentration equal to that used in the drug loading step, preventing drug loss during storage by diffusion to the storage solution. To assess the effect on the MFX release profile of long soaking/storage times in an MFX solution, a storage study was undertaken employing the most promising system. For that purpose, MFX-loaded, modified discs (discs #4, Scheme 1) were stored in an MFX solution with the same concentration as that used for loading them (5 mg/mL in HBSS), for periods of time ranging from 24 h up to 3 months, at room temperature. The obtained release profiles (Figure 6C) showed that no drug was lost, since none of the curves was below the curve for unstored samples (open circles, Figure 6C). In general, an increase in storage time in the MFX solution

lead to an increase in the amount of MFX released. Discs stored for 3 months showed no plateau during the 15 days of the release study and reached an amount of released MFX of $\sim 12.5 \mu\text{g}$ of MFX/mg dry disc. Additionally, there was no clear evidence of attainment of loading equilibrium during the time period of this study (3 months), since the curve for the 3-month storage was not clearly above the curve immediately below, due to its large error bars. It is not clear what was the reason for this large variation in the discs stored for 3 months.

The increase in released drug with storage time should be related to increased drug loading. The reason for the increase in the release duration of stored systems is not clear. One possible explanation would be that a very long loading time would allow drug molecules to reach much deeper into the disc and these molecules would take longer to make the reverse path, i.e., to reach the disc surface and be released. Provided that the amount of drug released in the initial burst is non-cytotoxic, this increase in released MFX with storage time, and the increase in the release duration, are valuable for the intended application.

3.4. Antibacterial activity evaluation

These studies were started by determining the MIC of MFX for *S. aureus* and *S. epidermidis*, through measurement of inhibition halos in agar diffusion assays. For both bacteria, the value of $0.25 \mu\text{g/mL}$ was obtained.

Microbiological tests were carried out to assess the efficacy of the MFX-loaded discs modified by Ar plasma-assisted grafting with HEMA in the presence of MFX (discs #4) against *S. aureus* and *S. epidermidis*. Two types of evaluations were performed: (i) evaluation of the activity of MFX released from drug-loaded discs during the release studies in the microfluidic cell, and (ii) evaluation of the activity of MFX-loaded discs by direct contact. The presence of inhibition halos in the tests carried out with the collected solutions, indicated that the drug released from the lenses under dynamic conditions, which are comparable to the *in vivo* situation, was still active against *S. aureus* and *S. epidermidis* (representative results for *S. aureus* are shown in Figure 7A and B). The concentration of MFX active against the microorganisms was estimated from the inhibition halos, using calibration curves previously determined. The concentration values obtained were lower than those obtained for the same solutions analyzed by HPLC (Table 2), indicating that a partial loss of activity of the drug against both bacteria occurred. Even though, the results show that the released MFX was active, at

least, until the last day of release tested (7th day) and that *S. aureus* is slightly more susceptible to MFX, since the inhibition zones for this bacteria were larger than those of *S. epidermidis* (results not shown). Direct contact tests, in which MFX-loaded, modified discs were placed directly in contact with the bacteria growing on the agar plates, demonstrated that the discs were active against the studied bacteria, although the obtained halos were too large to allow the quantification of the released drug (Figure 7C).

3.5. Cytotoxicity evaluation

With the aim of evaluating the cytotoxicity of the adopted controlled release system, the cytotoxicity of unmodified discs and of discs modified by Ar plasma-assisted grafting with HEMA, either loaded with MFX (discs #4; Scheme 1) or unloaded, was evaluated by direct contact with cells which are relevant for the intended application (CEC). CECs were seeded in 96-well plates at the same initial density of 2×10^4 cells/well, in the presence or absence of a disc. For each incubation time selected (1, 2 and 5 days), cell adhesion and proliferation was monitored by optical microscopy. As can be observed in Figure 8A, cells in contact with all discs were able to proliferate for, at least, 5 days, in a manner similar to those not in contact with a disc (negative control, K-), and both showed identical morphology. As such, there was no indication of detrimental effects on cell morphology and proliferation due to contact with either unmodified discs, discs modified by plasma-assisted grafting with HEMA or discs modified by plasma-assisted grafting with HEMA and loaded with MFX.

Cell viability in the presence of the discs was assessed through the MTS assay, which allows quantification of metabolically active cells. This assay is based in the reduction of a soluble tetrazolium salt (MTS) to a soluble, colored formazan product, in the presence of an electron coupling agent (PES) [51], thought to occur by NADPH or NADH produced by dehydrogenase enzymes of metabolically active cells. The obtained results (Figure 8B) showed that for modified, MFX-loaded discs, a small decrease in cell viability of 9 and 14%, relative to the negative control, occurred on the 1st and 2nd days of incubation, respectively, but was not detected on the 5th day of incubation. Thus, for 5 days, there were no signs of an overt decrease in cell viability after contact with modified discs or with modified discs releasing MFX. In addition, the decrease in cell viability after exposure to any of the discs was always much below the threshold for a cytotoxic effect (30%, according to the ISO 10993-5:2009 standard

[39]). As such, this MFX controlled release system (disc #4) seems promising for use as an MFX-releasing system for prophylaxis of postoperative endophthalmitis.

4. Conclusions

In the present work, surface modification of a polymethacrylate-based material used in the preparation of IOLs was selected as an approach to create a controlled drug release system to deliver MFX. This system aims to be used in the prophylaxis of postoperative endophthalmitis, an inflammation of the aqueous and vitreous humors of the eye which may occur after cataract surgery or after any type of surgical intervention in the eye. As such, this system is expected to release MFX above the MIC for bacteria causing this condition for, at least, 10 days.

The systems studied in this work were based on surface modification of discs by grafting of a PHEMA coating, employing Ar plasma-assisted grafting with HEMA in the presence of MFX and a final drug loading step by soaking in an MFX solution. The main aim of this coating was to create a barrier to the release of the drug, in order to achieve an extended delivery, without compromising the bulk properties of the material. After modification of the discs, a coating could be detected by contact angle goniometry, ellipsometry, AFM and SEM. Additionally, wettability, water content, visible light transmittance and the refractive index of the starting material, which are important parameters for the intended application (IOL), were not significantly affected by the coating. Unmodified discs loaded by soaking released reduced amounts of drug for ~24 h, under sink conditions, which was insufficient for the intended application.

Surface modification of the discs by Ar plasma-assisted grafting with HEMA in the presence of MFX, with a subsequent drug-loading step with MFX, resulted in a fourfold increase in the released MFX and an increase in the release duration to ~10 days, a release time which was sufficient for the intended application. These results were confirmed under hydrodynamic conditions equivalent to those of the aqueous humor of the eye. Under these conditions, this system released MFX at a concentration above de MIC for *S. aureus* and for *S. epidermidis* for ca. 12 days. A study of the drug release profiles of HEMA-modified discs stored in the MFX solution used for loading, for periods ranging from 24 h up to 3 months, showed that the amount

of drug released increased twofold in relation to the non-stored system, with the release duration extended to ca. 15 days.

Antibacterial activity evaluation showed that the released MFX was active against two pathogens usually associated with endophthalmitis (*S. aureus* and *S. epidermidis*), and cytotoxicity evaluation employing relevant cells (corneal endothelial cells), showed no cytotoxicity, although a small decrease in cell viability occurred. As such, the proposed system – IOLs modified by argon plasma-assisted grafting with HEMA in the presence of MFX, followed by loading of MFX by soaking – represents a promising candidate for use in postoperative endophthalmitis prophylaxis.

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Table 1. Water contact angle (WCA), equilibrium water content (EWC), refractive index (n) and roughness (R_a) of discs before and after modification by Ar plasma-assisted grafting with HEMA, together with the thickness of the coating determined by three different methods. Results are expressed as mean \pm SD.

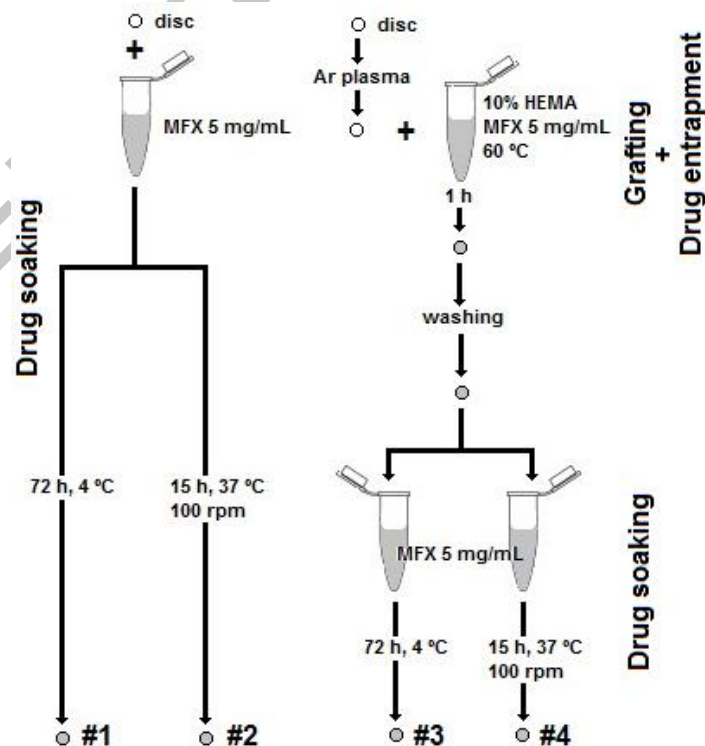
| | WCA, dry (°) | WCA, hydrated (°) | EWC (%) | n | Thickness (nm) | | | R_a (nm) |
|-----------------|--------------------|--------------------------|------------------------------|---------------------------------|----------------|----------------|----------------|---------------|
| | | | | | Ellipsometry | SEM | AFM | |
| Unmodified disc | 78 \pm 3** | 46 \pm 4 ^{ns} | 26 \pm 1 ^{ns} | 1.498 \pm 0.001 ^{ns} | – ¹ | – ¹ | – ¹ | 12 \pm 2 |
| Modified disc | 74 \pm 3** | 48 \pm 3 ^{ns} | 26.2 \pm 0.4 ^{ns} | 1.50 \pm 0.01 ^{ns} | 52 \pm 5 | 73 | > 45 | 34 \pm 2 |

** $p < 0.01$ for the difference modified/unmodified; $n = 9 - 12$; ^{ns} $p > 0.05$ for the difference modified/unmodified; $n = 10$ (two-tailed, unpaired t test, at a confidence level of 95%;).

¹ Not applicable.

Table 2. Moxifloxacin concentration ($\mu\text{g/mL}$) in the solutions collected at the 3rd, 4th and 7th day in the microfluidic assay carried out with MFX-loaded discs modified by Ar plasma-assisted grafting with HEMA in the presence of MFX (discs #4, Scheme 1). Values were determined by HPLC and from the microbiological tests with *S. aureus* and *S. epidermidis*.

| Day | HPLC [MFX] ($\mu\text{g/mL}$) | Microbiological assay [MFX] ($\mu\text{g/mL}$) | |
|-----|------------------------------------|---|-----------------------|
| | | <i>S. aureus</i> | <i>S. epidermidis</i> |
| 3 | 4 \pm 1 | 1.6 \pm 0.1 | 0.95 \pm 0.02 |
| 4 | 3 \pm 1 | 1.2 \pm 0.3 | 0.95 \pm 0.04 |
| 7 | 1.5 \pm 0.1 | 0.78 \pm 0.03 | 0.7 \pm 0.1 |



Scheme 1. Illustration of the preparation of the different types of MFX-loaded discs employed in this study (discs #1 to #4).

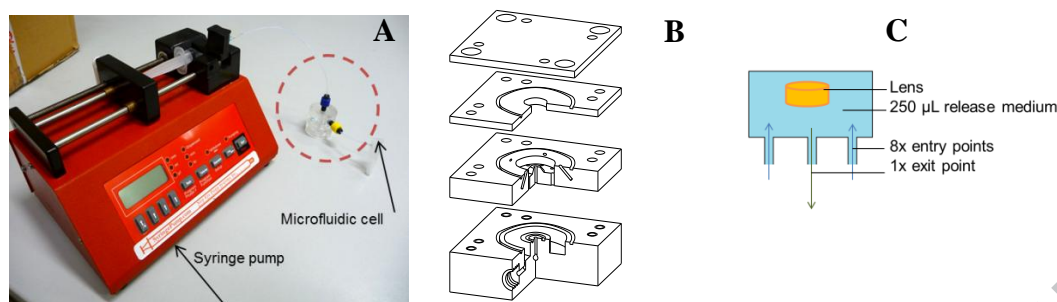


Figure 1. Experimental apparatus used in the dynamic release experiments. A – Microfluidic cell and syringe pump; B – Schematic representation of the microfluidic cell; C – Schematic representation of the inner chamber of the microfluidic cell.

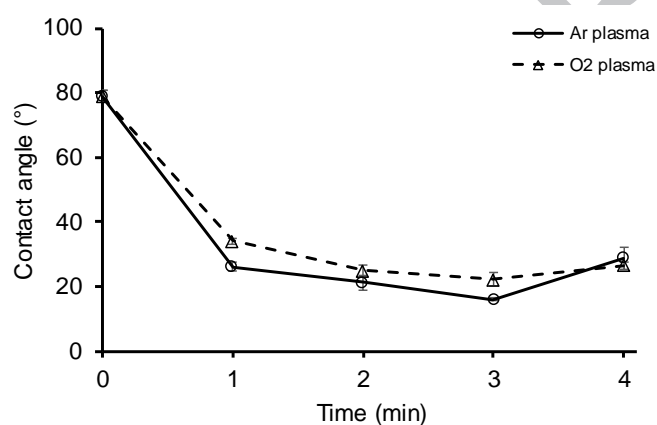


Figure 2. Variation of the water contact angle (sessile drop) of discs with the time of exposure to Ar or O₂ plasma. Results are expressed as mean \pm SD (5 drops per disc). The lines shown are only guides to the eye.

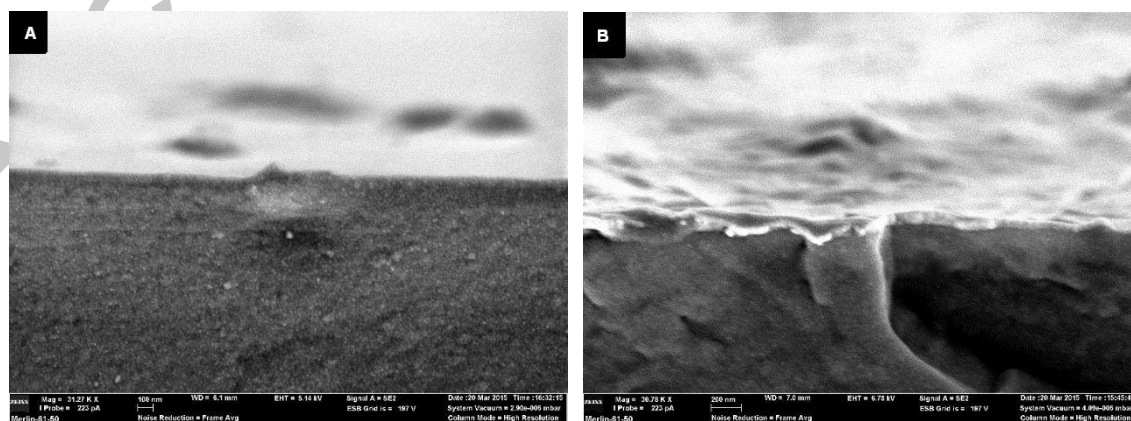


Figure 3. SEM images of cross sections of unmodified discs (A) and discs modified by Ar plasma-assisted grafting with HEMA (B). Scale bars: A – 100 nm; B – 200 nm.

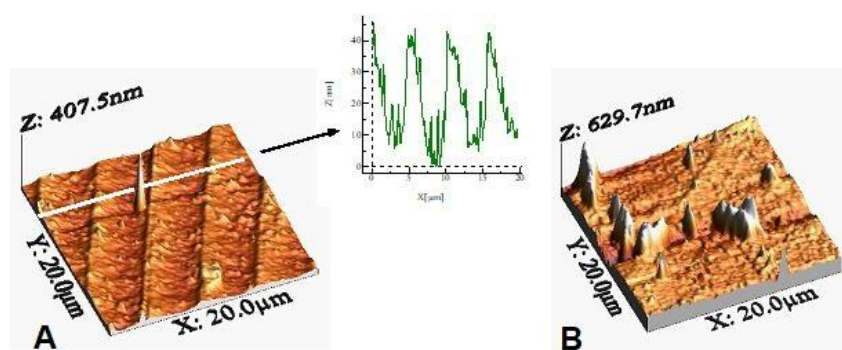


Figure 4. AFM images of unmodified discs (A) and discs modified by Ar plasma-assisted grafting with HEMA (B). The profile shown corresponds to the white line indicated in A.

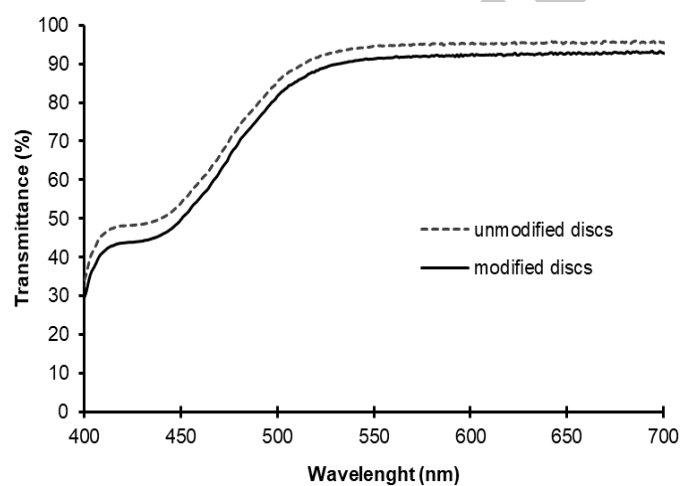


Figure 5. Transmittance spectra of discs in the visible region before and after Ar plasma-assisted grafting with HEMA.

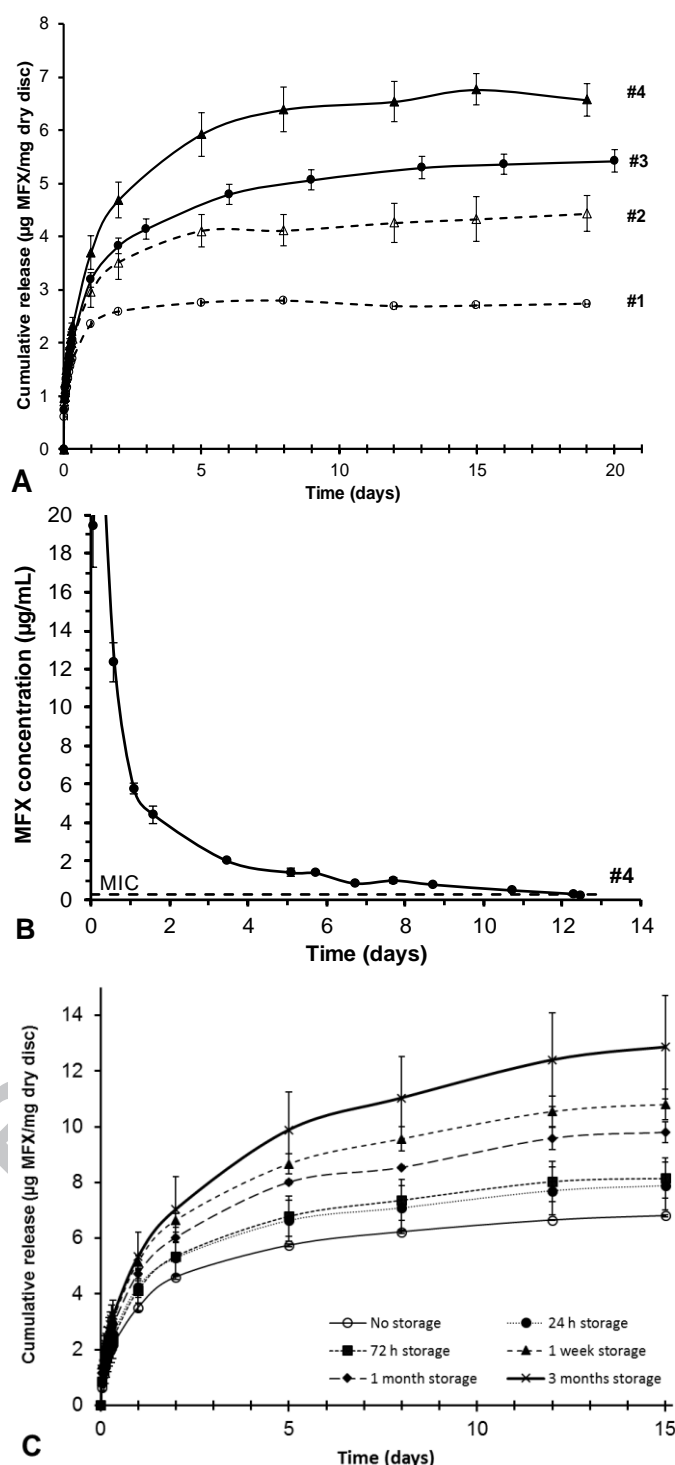


Figure 6. MFX release profiles for modified and unmodified discs under sink conditions, in batch mode (A), hydrodynamic conditions (B), and under sink conditions after storage in the MFX solution (C). Results are expressed as mean \pm SD ($n = 3$). The lines shown are only guides to the eye.

A. MFX release profiles (sink conditions) of discs loaded with MFX by soaking under two different conditions. #1 and #3 – Discs loaded for 72 h at 4 °C, unmodified and modified by Ar plasma-assisted grafting with HEMA, respectively; #2 and #4 – Discs loaded for 15 h at 37 °C, under shaking at 100 rpm, unmodified and modified by plasma-assisted grafting with HEMA, respectively. Dashed lines refer to unmodified discs; solid lines refer to modified discs.

B. MFX concentration release profile for the best system (discs #4), obtained under conditions similar to the hydrodynamic conditions in the eye. MIC – Minimal inhibitory concentration for *S. aureus* and *S. epidermidis* (0.25 $\mu\text{g/mL}$), represented by the dashed line.

C. MFX release profiles of discs #4 stored in the loading solution (250 μL of a 5 mg/mL solution of MFX in HBSS) for different time periods (sink conditions).

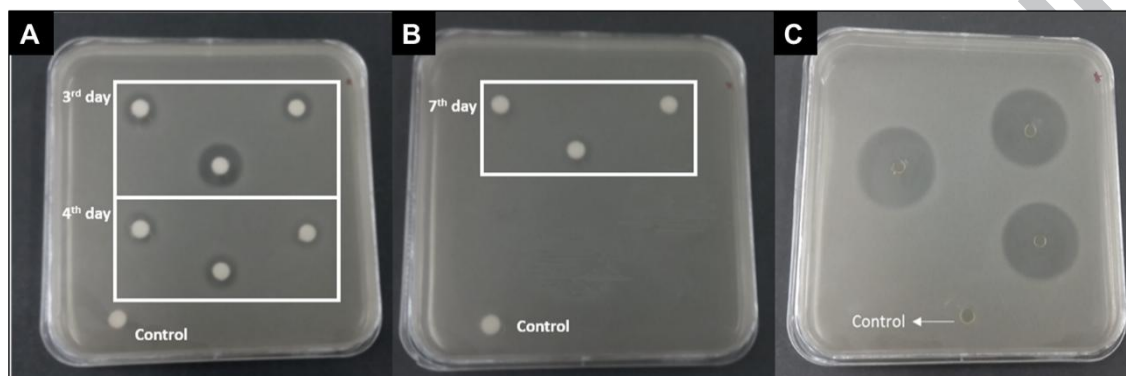


Figure 7. Inhibition halos for *S. aureus* obtained with MFX released from MFX-loaded discs modified by Ar plasma-assisted grafting with HEMA in the presence of MFX (discs #4, Scheme 1), in the case of solutions collected from the microfluidic assay at the 3rd and 4th day (A) and on the 7th day (B) of the drug release microfluidic study, and by direct contact (C).

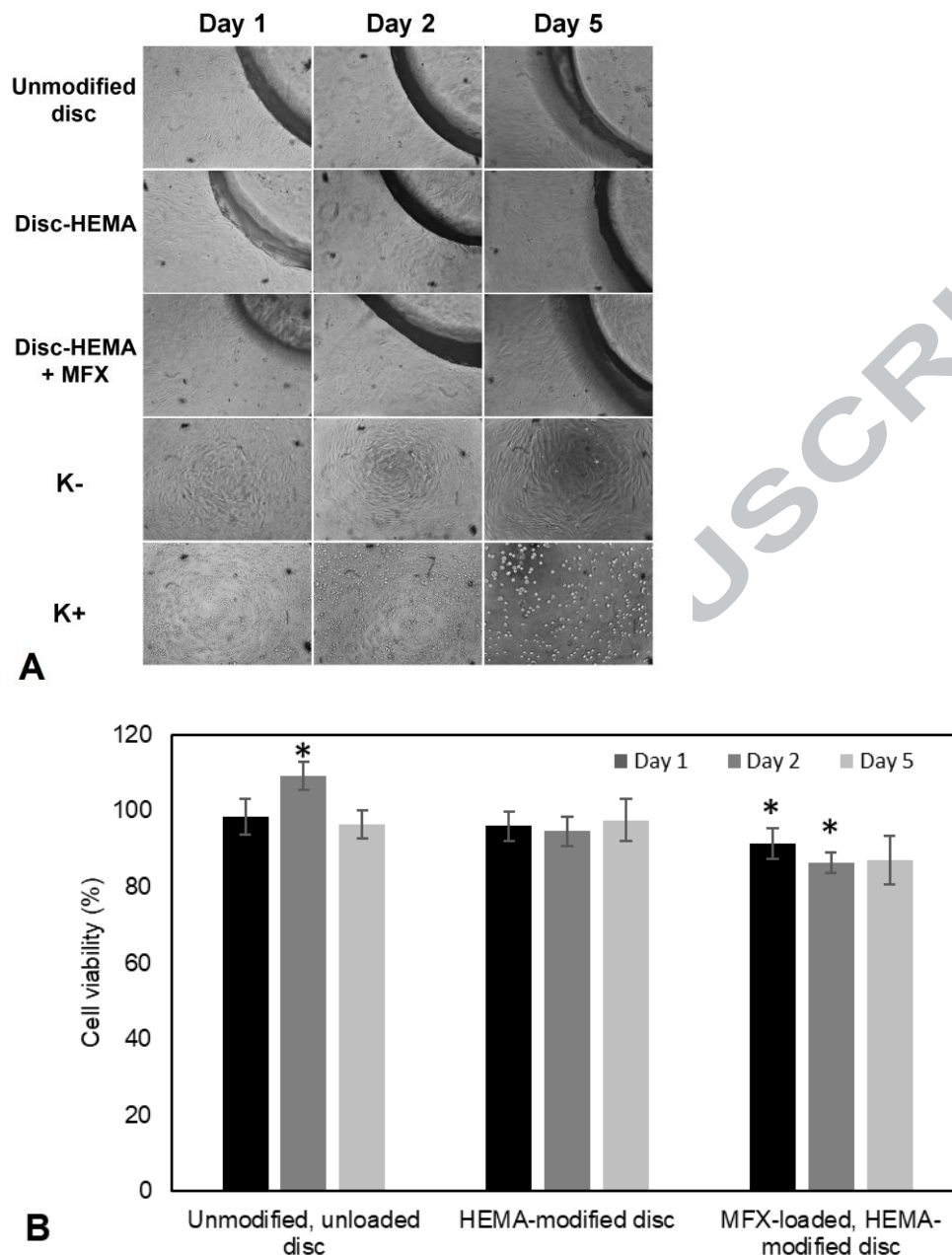


Figure 8. Results of the cytotoxicity evaluation of the best system (discs #4, Scheme 1), employing rabbit corneal endothelial cells (CECs). Cells were seeded at the same initial density, in the presence or absence of a disc.

A. Representative micrographs of CECs grown in the presence or absence of modified or unmodified discs, after 1, 2 and 5 days.

B. Cell viability of CECs after 1, 2 and 5 days in the presence or absence of modified or unmodified discs, measured by the MTS assay. Results are expressed as percentages in relation to the negative control, K- (mean \pm SD, $n = 4$).

* – $p < 0.05$ for comparisons of each sample with the negative control of the same day (one-way ANOVA followed by Dunnett's multicomparison test, at a confidence level of 95%).

HEMA-modified disc – Discs modified by Ar plasma-assisted grafting with HEMA, without MFX.

MFX-loaded, HEMA-modified disc – Discs modified by Ar plasma-assisted grafting with HEMA and loaded with MFX (discs #4, Scheme 1).

K- – Negative control (cells grown in the absence of a disc).

K+ – Positive control (cells grown in the absence of a disc and deliberately killed with ethanol).

Graphical abstract

