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Contact lens-based atropine delivery systems for myopia management: design and *in vitro-in vivo* evaluation☆

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ABSTRACT

The prevalence of myopia is increasing exponentially, particularly among children. Alongside other interventions, atropine can slow or even reverse myopia progression; however, topical eye drops produce short-lived concentration peaks that may cause systemic side effects while failing to ensure sustained ocular exposure. Existing contact lens (CL)-based delivery approaches present important limitations, including excessively rapid release, overly prolonged release, or low drug loading. This study aimed to address these shortcomings by designing CLs tailored for atropine delivery during day/night wearing. Hydrogels were synthesized by combining the anionic monomers methacrylic acid (MAA) and 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPSA) with the hydrophobic monomer benzyl methacrylate (BzMA) in specific ratios to modulate affinity for atropine. The resulting materials exhibited suitable solvent uptake, light transmittance, mechanical properties, and oxygen permeability. Anionic monomers enhanced atropine loading, while hydrophobic domains enabled more controlled release. *In vivo* testing showed that optimized formulations provided sustained drug levels in tear fluid and higher ocular tissue concentrations compared with eye drops. Ocular compatibility was confirmed both *in ovo* (HET-CAM test) and *in vivo*. For comparison, atropine loading and release from multifocal CLs designed for myopia control were also evaluated, and *in vitro* and *in vivo* correlations were investigated. Overall, these customized CLs represent a promising approach to integrate optical correction with sustained atropine delivery for myopia management.

1. Introduction

Myopia has emerged as a significant worldwide public health concern [1]. This refractive error causes visual images to focus in front of the retina leading to impaired distant vision. It is highly prevalent in developed countries, where it has reached epidemic proportions, and without control interventions, is expected to affect half of the world's population by 2050 [2]. The primary concerns associated with myopia include its progressive nature and the strong relation between high myopia and ocular diseases, which can lead to severe complications such as macular degeneration, retinal detachment, cataract, open-angle glaucoma, and even blindness [3]. Recent studies also suggest a trend toward an earlier age of onset, giving myopia more time to progress [4,5].

Myopia management should take a forward-looking approach, not only by correcting the refractive error but also preventing its progression [6]. In recent years, different approaches with distinctive mechanisms of action have been introduced into clinical practice, including environmental, optical and pharmacological interventions [7].

Optical interventions include myopia under-correction, bifocal and multifocal spectacles, gas permeable contact lenses (CLs), bifocal and multifocal soft CLs, and overnight-wear orthokeratology (Ortho-K) [8,9]. Multifocal soft CLs, like Misight® or Esencia®, correct the central refractive error while inducing a peripheral myopic defocus to inhibit eye growth. Differently, Ortho-K consists of personalized CLs, like Paragon CRT®, to temporarily reshape the cornea by redistributing epithelial and stromal cells. Even though Ortho-K is a safe and effective intervention with no minimum recommended age, its expected results can be affected by factors such as the age of myopia onset, pupil size, and degree of baseline myopia, among others [10].

From a pharmaceutical perspective, numerous drugs with different action mechanisms are being tested at various preclinical and clinical

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stages. These include adenosine receptor antagonists (7-methylxanthine), non-steroidal anti-inflammatories (ketorolac tromethamine), experimental agents (BHVI2), anticholinergics (tropicamide) and antimuscarinics (pirenzepine, atropine). Atropine topical instillation (low or medium doses eye drops) either alone or in combination with other approaches is so far the most effective treatment for myopia control and has become the current standard treatment on an off-label basis in many countries [7,11,12].

Combination therapy is gaining increased attention as means of optimizing first-generation treatments that have already demonstrated efficacy [13]. The rationale behind this approach is that targeting multiple mechanisms simultaneously may produce additive or synergistic effects. Examples include atropine eye drops combined with Ortho-K or retinal defocus [14–19].

Atropine base (Fig. 1) is a moderately lipophilic, low-molecular-weight drug (289.4 g/mol) with limited water solubility [20]. To increase its solubility, atropine is typically formulated as a sulfate salt, in which two atropine molecules are associated with one sulfate ion. However, atropine eye drops for myopia control face inherent limitations associated with topical ocular administration, including low ocular bioavailability (<5 %), increased side effects, poor treatment adherence, and significant variability in drug concentration on the ocular surface over time. To address these drawbacks, contact lens (CL)-based delivery systems have been investigated using several approaches [21,22].

Commercial soft CLs can absorb atropine sulfate after soaking in drug solutions (1–10 mg/mL), but they lack release control and typically exhibit burst delivery [23]. More recently, microemulsions of atropine base have been used to soak commercially available CLs, which achieved drug loadings of only ~ 4 μg per lens, with most of the drug released within 2 h. Importantly, the microemulsion components were found to be toxic to human corneal epithelial cells [24]. Other strategies have included adding atropine directly to CL monomers before polymerization [25] or incorporating it into implants embedded within silicone-based CLs [26], both resulting in overly prolonged release profiles that extend well beyond typical daily wear. Additional approaches involve modifying silicone hydrogels with anionic phenylboronic acid to enhance wettability and drug affinity [27], or attaching atropine to commercial Etafilcon A CLs via esterification, which achieved only limited loading (4–7 μg drug/CL) [28]. Overall, existing approaches either release atropine too rapidly or prolong its release excessively, highlighting the need for materials that provide a balanced, sustained release profile during 6–8 h wearing.

While the sulfate salt form of atropine increases hydrophilicity and allows the preparation of aqueous eye drops without additional solubilizing agents, this same property reduces its affinity for and retention within polymeric hydrogel networks. The central hypothesis and novelty of the present study is that CLs incorporating specific combinations of anionic and hydrophobic monomers—capable of interacting with the quaternary amine and benzyl groups of atropine sulfate, respectively—can enhance drug loading and enable sustained release. Moreover, incorporating atropine into a multifocal soft CL designed for myopia control may further improve therapeutic efficacy through additive or synergistic effects, provided efficient drug loading is achieved.

It should be noted that CL materials must meet specific requirements, including adequate water content, sufficient oxygen permeability, and high transparency to visible light, which are typically achieved through careful monomer selection. To satisfy these criteria, this study focuses on soft hydrogel CLs composed primarily of 2-hydroxyethyl methacrylate (HEMA) and silicone hydrogel CLs made of monomethacryloxypropyl-*sym*-polydimethylsiloxane hydroxypropyl-terminated (MCS-MC12) (Fig. 1), both of which have previously demonstrated their suitability [29,30]. However, copolymerization with anionic and hydrophobic monomers can alter the physicochemical properties of the CLs, so the effects of varying monomer proportions must be carefully evaluated [31].

Accordingly, the aim of this work was three-fold: (i) to design, for the first time, atropine-tailored CLs by incorporating anionic (methacrylic acid, MAA, and 2-acrylamido-2-methyl-1-propanesulfonic acid, AMPSA) and hydrophobic (benzyl methacrylate, BzMA) monomers in varying proportions to enable the loading and sustained release of therapeutically relevant amounts of atropine; (ii) to evaluate the atropine loading capacity and release profiles of various Esencia® multifocal CLs intended for myopia control; and (iii) to compare atropine-loaded CLs with eye drops in terms of drug accumulation in ocular tissues and to explore potential *in vitro-in vivo* (IVIV) correlations. To the best of our knowledge, this is the first study to rationally design both HEMA- and silicone-based CLs for sustained atropine delivery and to assess the drug's loading and release behavior in commercial CLs specifically approved for myopia management. Furthermore, a comprehensive *in vivo* assessment was conducted to elucidate the benefits of atropine-releasing CLs over eye drops, with emphasis on drug distribution in tear fluid and target ocular tissues.

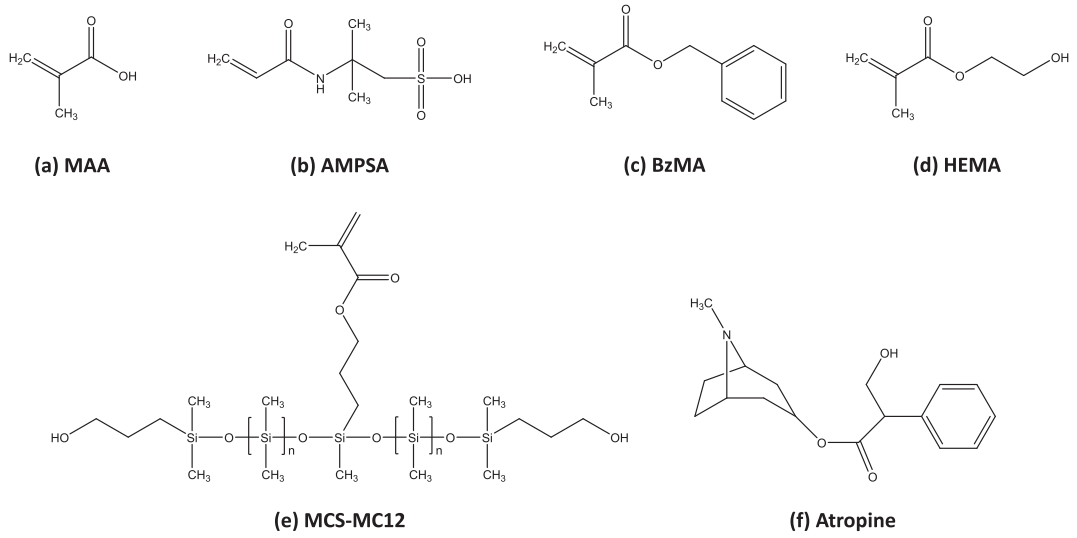

Fig. 1. Chemical structure of the monomers involved in the synthesis of HEMA- and silicone-based hydrogel contact lenses (a) methacrylic acid (MAA), (b) 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPSA), (c) benzyl methacrylate (BzMA), (d) 2-hydroxyethyl methacrylate (HEMA), (e) monomethacryloxypropyl-*sym*-polydimethylsiloxane hydroxypropyl terminated (MCS-MC12), and (f) atropine base.

2. Materials and Methods

2.1. Materials

2-Hydroxyethyl methacrylate (HEMA, 99 %) was purchased from Merck KGaA (Darmstadt, Germany). Benzyl methacrylate (BzMA, >95 %) was from Polysciences Inc. (Warrington, UK). Mono-methacryloxypropyl-*sym*-polydimethylsiloxane hydroxypropyl terminated (MCS-MC12, >95 %) was from Gelest, Inc (Morrisville, USA). 2-Acrylamido-2-methyl-1-propanesulfonic acid (AMPSA, 99 %), ethylene glycol dimethacrylate (EGDMA, 98 %), methacrylic acid (MAA, 99.5 %), dichlorodimethylsilane (99.5 %), 2,2′-azobis(2-methylpropionitrile) (AIBN, 98 %) and sodium dodecyl sulfate (95 %) were from Sigma-Aldrich (Steinheim, Germany). Atropine sulfate monohydrate (MW 694.83 g/mol; 97 %) was from Saurav Chemicals Limited (Punjab, India). Dimethyl sulfoxide (DMSO, 99.5 %) was from Fisher Scientific (Loughborough, UK). Potassium di-hydrogen phosphate (KH_2PO_4, 99.5 %) was from PanReac Química S.L.U. (Barcelona, Spain). Sodium chloride (NaCl, 99 %) was from Labkem (Barcelona, Spain). Acetonitrile for HPLC LC-MS grade and orthophosphoric acid 85 % were provided by VWR Chemicals (Fontenary-sous-Bois, France). Ultrapure water (resistivity > 18.2 MΩ·cm) was obtained by reverse osmosis (Milli-Q, Millipore Ibérica, Madrid, Spain). Schirmer test strips were from Contactcare Ophthalmics and Diagnostics (Guajarat, India). Propofol (Propofol Lipuro® 10 mg/mL) was supplied by Braun (Barcelona, Spain), and pentobarbital sodium (Exagon® 400 mg/mL) was from Richter Pharma AG (Wels, Germany). Esencia® contact lenses were supplied by Tiedra Farmacéutica, S.L. (Madrid, Spain).

2.2. Synthesis of HEMA- and silicone-based hydrogels and CLs

HEMA- and silicone-based hydrogels were prepared combining the monomers shown in Fig. 1, as summarized in Table 1. DMSO was used to dissolve AMPSA, and EGDMA and AIBN were added later as crosslinker and initiator, respectively. Once AIBN was completely dissolved (150 rpm, 30 min), the obtained monomer solutions were injected into pre-silanized glass molds (12 x 14 cm) separated by a 0.2 mm Teflon frame, and polymerized at 50 °C for 12 h and 70 °C for 24 h more. After polymerization, the obtained hydrogel sheets were demolded, boiled to remove most traces of unreacted monomers, and cut into 10- and 16-mm diameter discs. The discs were further washed at room temperature under stirring (350 rpm) in 0.5 L of water and NaCl 0.9 % solution for the complete removal of any residual monomers. The process was monitored by spectrophotometry (UV–Vis spectrophotometer Agilent 8453, Waldbronn, Germany). Finally, all hydrogel-discs were dried at 70 °C for 24 h and stored at room temperature until being used in further experiments (mean weight 18 ± 1 mg).

The most promising formulations (B3x and S2x) were also synthesized as CLs (**Fig. S1**) by adding 60 μL of the corresponding monomer solutions into curved polypropylene molds as previously described [29,31]. To ensure complete polymerization, AIBN amounts (0.0164 and 0.0246 g for B3x and S2x, respectively) and polymerization conditions (50 °C 12 h and 70 °C 30 h) were slightly adjusted. After polymerization, the CLs were demolded, boiled and washed as previously described, then dried at 40 °C for 2 h followed by 70 °C for an additional 2 h before being used in further experiments. Commercially available CLs (Table 2) were similarly washed in 1 L of purified water to remove the saline packing solution and dried using the same procedure prior to use.

Table 1
HEMA-based (H1-H5, B1-B5, B1x-B3x) and silicone-based (S1-S5, S1b-S3b, S1x-S3x) hydrogel compositions. AIBN (8.2 mg) was added to all compositions.

Code	HEMA (mL)	MAA (μL)	AMPSA (g)	EGDMA (μL)	DMSO (mL)	BzMA (μL)	MCS-MC12 (mL)
H1	4	–	–	6.04	0.5	–	–
H2	4	34.4	–	6.04	0.5	–	–
H3	4	68.9	–	6.04	0.5	–	–
H4	4	–	0.083	6.04	0.5	–	–
H5	4	–	0.166	6.04	0.5	–	–
B1	4	–	–	6.04	0.5	141.0	–
B2	4	34.4	–	6.04	0.5	141.0	–
B3	4	68.9	–	6.04	0.5	141.0	–
B4	4	–	0.083	6.04	0.5	141.0	–
B5	4	–	0.166	6.04	0.5	141.0	–
S1	3.5	–	–	12.08	0.5	–	0.5
S2	3.5	34.4	–	12.08	0.5	–	0.5
S3	3.5	68.9	–	12.08	0.5	–	0.5
S4	3.5	–	0.083	12.08	0.5	–	0.5
S5	3.5	–	0.166	12.08	0.5	–	0.5
B1x	3.75	–	–	12.08	–	282.0	–
B2x	3.75	34.4	–	12.08	–	282.0	–
B3x	3.75	68.9	–	12.08	–	282.0	–
S1b	3.5	–	–	12.08	–	–	0.5
S2b	3.5	34.4	–	12.08	–	–	0.5
S3b	3.5	68.9	–	12.08	–	–	0.5
S1x	3.5	–	–	12.08	–	250.0	0.25
S2x	3.5	34.4	–	12.08	–	250.0	0.25
S3x	3.5	68.9	–	12.08	–	250.0	0.25

2.3. Hydrogels characterization

2.3.1. Solvent uptake

The solvent uptake of all dried hydrogels and CLs was determined, in triplicate, in 4 mL of NaCl 0.9 % solution as the relative increase in weight before (W_0) and after immersion (W_t) at different time points (0.5, 1, 2, 4, 6 and 24 h), using Equation (1).

$$\text{Solvent uptake } (\%) = \frac{W_t - W_0}{W_0} \times 100 \quad (1)$$

2.3.2. Light transmittance

The transmittance (%) of the swollen hydrogels and CLs was recorded spectrophotometrically (Agilent Cary 60 UV–Vis spectrophotometer, Waldbronn, Germany) from 190-800 nm with scanning steps of 1 nm. The measurements were immediately carried out after the swelling (n = 2).

2.3.3. Mechanical properties

Wide strips (9-mm) of hydrogels and CLs previously hydrated in NaCl 0.9 % were fixed to the upper and lower clamps of a TA.XT Plus Texture

Table 2
Commercial CLs used, including material classification and monomer composition. Refractive powers measured in diopters (D) are specified as follows: −3.00 and −5.00.

CL name	Code	Material classification	Monomer composition
Esencia® 50 % −3.00	E 50 % −3.00	Filcon II2 (Acolficon B)	NVP [f], MMA [c], HEMA [a]
Esencia® 50 % −5.00	E 50 % −5.00	Filcon II2 (Acolficon B)	NVP, MMA, HEMA
Esencia® 59 % −3.00	E 59 % −3.00	Filcon II2 (Acofilcon A)	NVP, MMA, HEMA
Esencia® 59 % −5.00	E 59 % −5.00	Filcon II2 (Acofilcon A)	NVP, MMA, HEMA
Esencia® Methafilcon A	ME −3.00	Filcon IV2 (Methafilcon A)	HEMA, DEG [e], MAA [b], EGDMA [d]
Esencia® Etafilcon A	EE −3.00	Filcon IV (Etafilcon A)	HEMA, MAA [b]
Esencia® Unisil	E Unisil −3.00	Filcon 5B	HEMA, NVP, [g]TRIS, [h]SIGMA

[a]2-hydroxyethyl methacrylate (HEMA), [b]methacrylic acid (MAA), [c]methyl methacrylate (MMA), [d]ethylene glycol dimethacrylate (EGDMA), [e]diethylene glycol (DEG), [f]N-vinyl-2-pyrrolidone (NVP), [g]3-methacryloxypropyltris(trimethylsiloxy)silane (TRIS), [h]3-(3-methacryloxy-2-hydroxypropoxy)propylbis(trimethylsiloxy)methylsilane (SIGMA).

Analyzer (Stable Micro Systems Ltd., Surrey, UK) fitted with a 5 Kg load cell. The Young's modulus was calculated using the slope of the initial linear portion of the tensile stress versus engineering strain curve obtained at a crosshead speed of 0.1 mm/s.

2.4. Ex-vivo atropine permeability tests

The permeability of atropine sulfate (1 mg/mL in water and in NaCl 0.9 %) through fresh porcine corneal and scleral tissues was first evaluated *ex-vivo* in triplicate, following a previously described protocol [32]. The eyes were sourced from a local slaughterhouse (Compostelana de Carnes S.L., Santiago de Compostela, Spain). Isolated corneas and scleras were mounted in vertical Franz diffusion cells (permeation area of 0.785 cm^2) and equilibrated with carbonate buffer pH 7.2 (37 °C, 400 rpm, 30 min). Then, the carbonate buffer in the donor chamber was replaced with 1 mL of the corresponding atropine solution, and the chamber was sealed with parafilm to avoid evaporation. At pre-established times (0.5, 1, 2, 3, 4, 5 and 6 h), 1 mL samples were taken from the receptor chamber (6 mL) filtered (PTFE hydrophilic syringe filter, 13 mm, 0.22 μm, Scharlab, Barcelona) and analyzed by HPLC. Each withdrawn sample was immediately replaced with the same volume of fresh carbonate buffer. The lag time, the apparent permeability coefficient (P_{app}) and the steady state flux (J) were calculated as previously indicated [33,34].

At the end of the experiment, the amounts of atropine remaining in the donor chamber and accumulated in the tissues were also quantified. Atropine was extracted as previously described [35], by placing the tissues in Falcon® tubes containing 3 mL of ethanol:water (50:50 v/v) that were kept at 37 °C under intense stirring until the next day, sonicated for 99 min at the same temperature and centrifuged (1000 rpm, 5 min, 25 °C). Then, the supernatant was filtered (PTFE syringe filter, 13 mm, 0.22 μm, Scharlab, Barcelona), centrifuged (14,000 rpm, 20 min, 25 °C) and filtered again to be analyzed by HPLC.

The amount of atropine in the samples was quantified using a JASCO (Tokyo, Japan) HPLC (AS-4140 Autosampler, PU-4180 Pump, LC-NetII/ADC Interface Box, CO-4060 Column Oven, MD-4010 Photodiode Array Detector), fitted with a C18 column (Waters XSelect HSS T3, 3.5 μm, 4.6 x 150 mm). The analysis was carried out by isocratic elution with a mobile phase of acetonitrile:buffer 30:70 v/v at a flow rate of 1 mL/min for 6 min. The buffer solution was prepared by dissolving 3.5 g of sodium dodecyl sulfate in 606 mL of a 7 g/L KH_2PO_4 solution (pH adjusted to 3.3 using phosphoric acid 5.8 g/L) to which 320 mL of acetonitrile were added. The mobile phase was filtered through a 0.22 μm nylon filter before being placed in the equipment. The injection volume was 80 μL, and the temperature column set to 30 °C. Validation was carried out at 210 nm using two different calibration curves of atropine in water, one in the 0.5–20 μg/mL range and another in the 20–70 μg/mL range. Retention time was ~ 3.7 min.

2.5. Atropine loading and release tests

The incorporation of atropine into the different formulations was carried out by the soaking method. First, dried (37 °C, 24 h) HEMA- and silicone-based hydrogel discs (Table 1) were weighed (n = 4) and placed into Falcon® tubes containing 5 mL of a 1 mg/mL atropine solution in water or NaCl 0.9 % solution. The loading was carried out at 25 °C and 180 rpm for 24 h in an incubating shaker (Incubator 1000, Heidolph, Germany), taking aliquots (300 μL) of the loading medium at pre-established times (2, 4 and 24 h). The pH of the loading solution was measured using a pH & Ion-meter (GLP2 22 Crison, Barcelona, Spain).

After 24 h, the loaded discs were rinsed and transferred to new Falcon® tubes containing 6 mL of NaCl 0.9 %. The release was carried out at 36 °C and 180 rpm for 24 h in an incubating shaker (Incubator 1000, Heidolph, Germany). At pre-set times (0.5, 1, 1.5, 2, 3, 6 and 24 h), 300 μL samples of release medium were taken and replaced with an equivalent volume of fresh NaCl 0.9 % solution. After the release test, the hydrogels were gently rinsed with water and transferred to Eppendorf® tubes containing 1 mL of water to extract the remaining atropine inside the polymeric network [29]. The extraction procedure was carried out at 36 °C and 180 rpm for 8 h in an incubating shaker (Incubator 1000, Heidolph, Germany). The total amount of atropine loaded into the hydrogels was determined by summing the amount released and the residual amount extracted after release, which was negligible.

The loading of the most promising CLs formulations (B3x, S2x, EE and ME) was also evaluated using atropine solutions at 0.1 and 0.2 mg/mL in NaCl 0.9 % and 0.8 % medium. The 0.2 mg/mL solution of atropine in NaCl 0.8 % was assayed considering the contribution of atropine to the osmotic pressure of the medium. By modifying the concentration of the NaCl solution we expected to maintain a certain hypotonicity that facilitates both the drug loading into the polymeric network and the penetration of the released drug into eye tissues [36,37]. In this instance, the loading was carried out by placing dried (37 °C, 24 h) CLs in 10-mL glass vials containing 5 mL (0.1–0.2 mg/mL atropine in NaCl 0.9 %) or 1 mL (0.2 mg/mL atropine in NaCl 0.8 %) of the corresponding loading solution, which were immediately after hermetically sealed and sterilized by steam heat (121 °C, 20 min; Raypa Steam Sterilizer, Terrasa, Spain). After sterilization, the vials were kept at room temperature for 96 h under magnetic stirring, and the release experiments were carried out as previously described, considering the following sampling times: 5, 15, 30 min, every hour until 8 h, and 24 h.

The amounts of atropine loaded and released were quantified using a Waters HPLC (Autosampler Waters 717, Waters Controller 600, Photodiode Detector 996, Milford, MA, USA) equipped with a C18 column (Waters XSelect HSS T3, 3.5 μm, 4.6 x 150 mm) and operated with the Empower2 software. The analysis was carried out by isocratic elution using a mobile phase of acetonitrile:buffer 30:70 v/v at a flow rate of 1 mL/min for 5 min. The buffer solution was prepared as previously described. The injection volume was 50 μL and the column was kept at 30 °C. The calibration curve was performed with standard solutions of atropine in NaCl 0.9 % (0.625 – 60 μg/mL) and the absorbance measured at 210 nm. Retention time was ~ 3.7 min.

The network/water partition coefficient ($K_{N/W}$) was estimated as the difference between the total amount loaded and the amount hosted in the aqueous phase, with respect to the atropine concentration in the loading solution [31].

2.6. HET-CAM test

The ocular compatibility of H hydrogel discs and the most promising CLs (B3x, S2x, EE and ME) was evaluated *in ovo* through the hen's egg test on the chorioallantoic membrane (HET-CAM) assay, as previously described [38]. After 8 days of incubation (37 °C, 60 % relative humidity), the eggshell of fertilized hen eggs (Coren, Spain) was opened, and the inner membrane was moistened with NaCl 0.9 % before being carefully removed to expose the chorioallantoic membrane (CAM). Replicates of the most promising CL formulations (B3x, S2x, EE and ME) previously loaded with 1 mL of a 0.2 mg/mL atropine in NaCl 0.8 % solution and steam-heat sterilized (121 °C, 20 min) were placed in separate on the CAM. The compatibility of the loading solution was also assessed by applying 300 μL of the same sterilized solution onto the CAM. The vessels were observed for 5 min, registering the times at which hemorrhage, vascular lysis or coagulation appeared to calculate the IS as previously described [39]. NaCl 0.9 % and NaOH 0.1 N solutions were used as negative and positive controls, respectively.

2.7. In vivo experiments: Atropine tear levels and ocular biodistribution

2.7.1. Study design and animal groups

All *in vivo* experiments fulfilled 3R's principles and were carried out in accordance with the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, the Spanish (RD53/2013) and

European (2010/63/EU) Directives, and under the Ethics Committee for Animal Experimentation of the Complutense University of Madrid (CEEA-UCM) [code O00023280e2100023620] and the Bioethics Committee of the University of Santiago de Compostela (CEEA-USC) and Xunta de Galicia [Registration number: ES150780292901] approvals. Both the USC and the UCM are adhered to the "Transparency Agreement on the Use of Animals in Scientific Experimentation", promoted by the European Animal Research Association (EARA) and the Spanish Federation of Scientific Societies (COSCE). Protocols previously described by our research group were followed with slight modifications [29,31,35].

Five different *in vivo* experiments were conducted with a total amount of 76 male New Zealand white rabbits, as shown in Fig. 2. According to standard clinical practice, two different concentrations of atropine solutions were tested, 0.1 mg/mL (in NaCl 0.9 %) and 0.2 mg/mL (in NaCl 0.8 % and NaCl 0.9 %). For each experiment, the animals were randomly assigned to two different groups: one group was treated with the corresponding atropine-loaded CLs (CLs group) while the other group was treated with a single eye drop (50 μL) of atropine in NaCl 0.8 % or 0.9 % solution (eye drops group) according to the CLs loading. The CLs were steam-heat sterilized, and the eye drop formulations were sterilized by filtration (polyethersulphone (PES) 0.22 μm syringe filter, Filter-Lab®, Barcelona, Spain). The study intervention was applied on the right eyes, and the left eyes served as controls. To minimize the effects of subjective bias, all experiments started around 8 a.m. in the morning. Each experiment was conducted over 3 to 5 days, depending on the number of animals, with four rabbits assigned to each day. Rabbits were randomly assigned to each day and treatment.

All animals (approx. 3 months age and an average weight of 3.16 ± 0.60 Kg) were stabled for one week before the beginning of the experiments in a light-controlled room with 12 h light–dark cycles inside individual cages with total access to food and water and environmental conditions of humidity and temperature around 60 % and 19 °C, respectively. Before performing the study, the overall health status of the animals was evaluated, and their eyes examined using a slit lamp VX75 slit-lamp (Luneau Technology, Chartres, France). No animals were discarded due to health conditions or eye damage. On the day of the experiment, the corresponding CLs (Fig. 2) were removed from the loading solution, rinsed with 5 mL of a sterile saline solution to withdraw the excess of loading medium and placed on the ocular surface of the right eyes of the CL group rabbits, without the use of local anesthesia. The eyes of the rabbits were manually closed every few minutes to avoid lens dehydration. The animals were euthanized after 1 h, 6 h and 8 h of the beginning of the experiment.

2.7.2. *Atropine levels in tear fluid*

In the eye drop group, a single drop (50 μL) of the corresponding atropine solution (Fig. 2) was instilled in the lower conjunctival sac of the right eye using a micropipette taking special care not to touch the ocular surface of the animals. A VX75 slit-lamp (Luneau Technology, Chartres, France) was used to observe the ocular surface of all rabbits at 0, 1 and 6 or 8 h, depending on the duration of the experiment, and the irritation score was assessed following the Draize criteria [40]. Moreover, the pupil diameter of both eyes of all animals was also monitored at times 0, 1, 4 and 6 h. The Draize test evaluations and pupil diameter measurements were performed independently by two researchers in parallel, with consensus reached on the final scores.

At pre-established times, 0, 5, 15, 30 min, and every hour until 1, 6 and 8 h depending on the experiment duration, samples of tear fluid were collected using Schirmer test strips placed in the tarsal conjunctiva of the lower eyelid for 10 s with closed eyes to avoid the reflex secretion associated with blinking. The volume collected was calculated checking the millimeters of wetted strip [41]. After collection, all moisturized Schirmer test strips were cut into small sections, placed into 1.5 mL Eppendorf® tubes containing 150 μL of NaCl 0.9 % and vortexed for 1 min to ensure atropine extraction. Then, the small strip pieces were carefully removed, and the tubes were stored, sealed with parafilm and protected from light at 4 °C until UPLC-MS analysis. Just before UPLC-MS analysis, all samples were vortexed again for 30 s, diluted 1.5 times with acetonitrile in a UPLC-well plate (80 μL of sample + 40 μL of acetonitrile) and mixed using an Automated Liquid Handling System, Caliper Zephyr, 3 cycles of 50 μL at 78 μL/s. After that, the plate was centrifuged at 3700 rpm and 4 °C for 30 min. Atropine quantification was carried out using a Waters Acquity UPLC H-Class coupled with a Xevo TQD MS System fitted with a BEH C18 column (1.7 μm 2.1 x 50 mm, Waters) at a flow rate of 0.6 mL/min and 35 °C. The analysis was performed by gradient elution using water + 0.1 % formic acid as solvent A, and acetonitrile + 0.1 % formic acid as solvent B. The gradient program used was as follows: 0–0.1 min. 5 % B, 0.1–1.0 min. 5–100 % B, 1.0–2.0 min. 100 % B, 2.0–2.1 min. 100–95 % B, and 2.1–2.5 min. 5 % B. Electrospray ionization (ESI) was run in positive mode with a source temperature of 150 °C and a desolvation temperature of 500 °C. Capillary voltage was set to 3 kV and the cone voltage was set to 45 V. Desolvation gas flow was 900 L/h and cone gas flow was set to 50 L/h. Atropine was monitored in multiple reaction monitoring (MRM) mode. 290.221 > 124.166 *m/z* transition was quantified.

At the end of the experiment, the CLs were carefully removed from the ocular surface, placed into empty 1.5 mL Eppendorf® tubes and stored at 4 °C to perform the extraction procedure as described in section 2.4.

The mean residence time (MRT) was determined by dividing the area under the first moment curve (AUMC; from the plot of concentration × time versus time) by the area under the concentration versus time curve (AUC). Both AUMC and AUC were calculated from experimental atropine concentrations in tear fluid using the trapezoidal rule [23,31].

In vitro-in vivo (IVIV) correlations were investigated using Levy plot analysis, where the percentage of drug released into the tear fluid at a given time was plotted against the corresponding percentage released *in vitro*. The *in vivo* release percentage of atropine was estimated using Eq. (2) [42], as follows

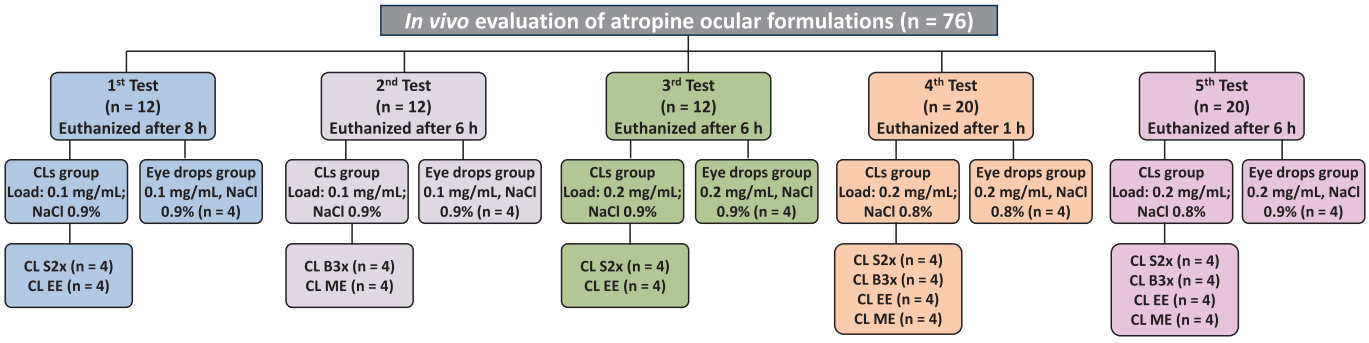

Fig. 2. Planning of the *in vivo* tests. Atropine eye drops and atropine-loaded CLs were in all cases evaluated in parallel. Drug concentration in the eye drops and the solutions used for the soaking of the CLs is indicated together with the number (n) of animals tested in each experiment.

$$\textit{Atropine released in vivo}\ (\%) = \frac{AUC_{0-t}}{AUC_{0-last}} x100 \quad (2)$$

Regression analysis was carried out using SigmaPlot for Windows v.14 software (Systat Sofware Inc., Germany).

2.7.3. Atropine levels in ocular tissues

At the end of the sampling period, all rabbits were euthanized by intravenous injection of a weight-based dosing combination of 7.5 mg/Kg of propofol (Propofol Lipuro® 10 mg/mL) and 200 mg/Kg of pentobarbital sodium (Exagon® 400 mg/mL). The aqueous humor was directly withdrawn from the anterior chamber using a 25 G needle. Then, the eyes were enucleated, and the different structures (cornea, crystalline lens, vitreous humor, retina and sclera) were separated, cut, weighed and placed in 1.5 mL Eppendorf® tubes to which, except for aqueous and vitreous humors, different volumes of NaCl 0.9 % were added (500 μL for the cornea and the crystalline lens, 200 μL for the retina and 800 μL for the sclera). All tubes were vortexed for 30 s before being kept overnight at 4 °C under soft stirring, and then, the protein denaturation process was carried out as previously described [29,31,35]. Briefly, the tubes were vortexed for 1.5 min and the scleral and corneal tissues were removed. To denature proteins, samples were heated at 98 °C for 2 min, cooled in an ice bath for 10 min and centrifuged at 13,000 rpm at 25 °C for 10 more minutes. The supernatants were collected and kept at 4 °C until being analyzed by UPLC-MS as described above.

2.8. Statistical analysis

Statistical analysis was conducted using Statgraphics Centurion 9 X64 (Statgraphics Technologies, Inc., Warrenton, VA, USA). Descriptive data are presented as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by a Multiple Range Test was performed, with the significance level set at 0.05. All results were considered, and no outliers were discarded.

3. Results and discussion

3.1. Synthesis of HEMA- and silicone-based hydrogels and CLs

Various HEMA- and silicone-based hydrogels and CLs were synthesized (Table 1) to evaluate the effect of different compositions on the loading and sustained release of therapeutically relevant amounts of atropine for myopia control. It is worth noting that any of the tested compositions could be molded to produce multifocal CLs for myopia management. Varying monomers mixtures of HEMA and a third-generation silicone macromer, i.e. monomethacryloxypropyl-*sym*-polydimethylsiloxane hydroxypropyl terminated (MCS-MC12), as structural monomers of the polymer network, were prepared including anionic monomers of different pKa, namely MAA of pKa = 4.65 and AMPSA of pKa = 1.67, and hydrophobic monomers as BzMA to meet two ground criteria: (i) the inclusion of adequate proportions of anionic and hydrophobic monomers able to interact with atropine and modulate its loading and release, and (ii) the development of biocompatible polymers that match the requirements for commercial CLs.

Atropine interacts in a competitive reversible manner with muscarinic receptors forming hydrogen bonds between its hydroxyl group and the binding site [43]. Besides the possibility to form hydrogen bonds, atropine's ability to interact with functional groups from the battery of selected co-monomers could be enhanced by the presence of hydrophobic regions and ionic interactions with the quaternary amine, similar to the ones created between acetylcholine and muscarinic receptors. For this reason, acrylic (MAA, BzMA) and sulfonic acid (AMPSA) co-monomers were combined with HEMA and MCS-MC12 at various proportions. BzMA was chosen to achieve an extended release profile due to its aromatic group that could strengthen hydrophobic interactions with atropine. One composition without functional anionic monomers was included in each set as control (H1, B1, S1, B1x, S1b, S1x). DMSO was required for a complete dissolution of AMPSA and included in all compositions of the same set (H, B, S) for comparative purposes. In the preliminary screening tests, the hydrogel discs were synthesized with a 0.2 mm thickness in order to resemble as much as possible the thickness of commercially available CLs.

According to the mean weight (~18 mg) of our designed hydrogels (H, B, S, Bx, Sb, Sx) and considering a molecular weight of atropine sulfate salt monohydrate of 695 g/mol, if each MAA (amount between 0.764–1.722 %; MW = 86.09 g/mol) or AMPSA (amount between 1.844–3.688 %; MW = 207.25 g/mol) monomer binds to an atropine molecule, the maximum amount of atropine that could be loaded would be 1110–2503 μg/g for MAA and 1113–2226 μg/g for AMPSA. These amounts would satisfy by far the actual clinical practice where a wide variety of atropine eye drops concentrations ranging from 0.1 mg/mL (3–5 μg) to 5 mg/mL (150–250 μg) have been assayed for the control of the progression of myopia [7].

In addition, considering the higher susceptibility of children to systemic atropine exposure in comparison with adults, the amounts of atropine loaded into the CLs and subsequently eluted should reflect a compromise between effectiveness and safety.

3.2. Hydrogels characterization

3.2.1. Solvent uptake

The solvent uptake of all formulations was evaluated at room temperature for at least 24 h and the results are shown in **Table S1** and **Fig. S2**. When immersed in NaCl 0.9 %, all discs and CLs rapidly sorbed the medium and reached the equilibrium within the first hour. Polymeric networks bearing polar groups as structural units of the polymer enable better interactions with water and thus higher values of solvent uptake. The similarities observed between B and S hydrogel discs could be related to the hydroxypropyl terminations of MCS-MC12 monomer. The incorporation of anionic monomers (MAA and AMPSA) increased the swelling degree, as the ionic groups make the network more hydrophilic. This relation was more remarkable for AMPSA, which led to a notable increase in the solvent uptake, especially for S4 (59.69 ± 0.32 %) and S5 (75.64 ± 0.23 %) hydrogels. This trend was also observed for B4 (51.33 ± 0.76 %) and B5 (58.18 ± 5.50 %) hydrogels, but to a lower degree, probably due to the incorporation of the hydrophobic BzMA monomer. The contribution of BzMA together with a higher concentration of crosslinker (EGDMA) to decrease the solvent uptake was confirmed for Bx and Sx series, with values of 39.53 ± 0.02 % for B3x, and 35.44 ± 1.10 % for S2x.

The highest values were observed for all Esencia® CLs with no differences between different refractive powers within the same material. Notably, Esencia® CLs are widely distributed worldwide, particularly those made of etafilcon A CLs (coded as EE in the present study) which have been on the market for 30 years and represent the most widely used composition for commercially available hydrogel CLs (HEMA + MAA; Table 2) [44]. Etafilcon A (coded EE) and Methafilcon A (coded ME) belong to Group IV CLs due to their content of the ionic monomer MAA. Acolficon A and B CLs are neutral Group II CLs but are prepared with the hydrophilic monomer NVP, which gives them a high capacity for water uptake (coded E 50 % and E 59%, Table 2 **and S1**). Overall, the values recorded for all compositions were in good agreement with the values reported for different conventional hydrogel and silicone hydrogel soft CL materials [44–46].

3.2.2. Light transmittance

All formulations showed light transmittance values above 90 % in the visible range (600 nm) after swelling in NaCl 0.9 % for at least 24 h. The recorded light transmission fulfilled the standard requirements for commercial CLs **(Fig. S3)**. Moreover, the decrease in light transmission below 380 nm in both Esencia® 59 % and Unisil® CLs could be justified

by the presence of UV blockers, which provide an efficient protection against UV light, without compromising visible range **(Fig. S3E)**.

3.2.3. Mechanical properties

Mechanical properties, especially Young's modulus, of CLs determine comfort, handleability and adequate fit [47]. The Young's modulus of all hydrogels **(Table S1)** was in good agreement with the average range for soft CLs, set between 0.3 and 1.9 MPa [48,49]. No significant differences between our developed materials and the supplied Esencia® CLs were observed. Silicone-based hydrogels showed increased Young's modulus compared to HEMA-hydrogels, something expected considering their higher content in siloxanes, but, in all cases, the stiffness was lower than for first-generation silicone hydrogel materials, more associated with mechanical complications [48]. In conventional HEMA-based hydrogels, water content has a major impact on mechanical properties [50]. This trend was also observed here for Esencia® hydrogel CLs where a higher water content caused a decrease in the Young's modulus, obtaining average values of 0.49 ± 0.09 MPa and 0.37 ± 0.04 MPa for E 50 % and E 59 %, respectively.

3.3. Atropine corneal and scleral permeability

The capability of atropine to permeate through the cornea and the sclera was evaluated using vertical diffusion Franz cells that mimic the penetration of substances from the surface to the anterior chamber of the eye. To perform the assay, porcine eyes were chosen as an ocular model according to their structural resemblance to the human eyes in terms of anatomical, histological, and physiological parameters [34,51,52]. The experiment was carried out by filling the donor chambers with 1 mL of an atropine solution (1 mg/mL) in water or NaCl 0.9 % medium. At the end of the experiment (6 h) atropine was detected in all receptor chambers, thus indicating its ability to diffuse through both tissues. The penetration of atropine occurred faster and to a greater extent through the sclera, especially when atropine was dissolved in water instead of NaCl 0.9 % likely due to an osmotic effect ($p < 0.001$) [36,37]. In fact, no atropine could be quantified in the corneal receptor chambers until the third hour of the test, as shown in Fig. 3.

The accumulated drug amounts in sclera were 123.13 ± 14.11 μg/cm^2 for atropine solution in water and 158.08 ± 19.01 μg/cm^2 for atropine solution in NaCl 0.9 % (the solvent did not cause significant differences; $p = 0.063$). In the case of cornea, accumulated amounts were lower with values of 28.13 ± 0.61 μg/cm^2 for atropine solution in water and 10.12 ± 1.44 μg/cm^2 for atropine solution in NaCl 0.9 % (the solvent caused significant differences; $p < 0.001$). Differences between cornea and sclera for each solution were also significant ($p < 0.001$). In line with these results, the atropine amounts that remained in the donor chambers were lower for the sclera (39.18 ± 1.48 μg in water and 42.19 ± 0.82 μg in NaCl 0.9 %) than those measured in the corneal tests (74.16 ± 26.76 μg in water and 56.80 ± 0.56 μg in NaCl 0.9 %) ($p < 0.001$). These findings are in good agreement with previous studies performed in white rabbits which evidenced that topically applied atropine easily penetrates the anterior structures of the eye [53,54].

The ocular penetration behavior of atropine may be explained by its use in the sulfate form, which significantly increases water solubility and molecular weight. These features facilitate a preferred penetration through the sclera given this tissue enables the pass of hydrophilic and more bulky compounds. However, despite the high molecular weight and the ionized state of atropine, the transcorneal route is still possible at some level due to the moderately lipophilic logP (~1.83) of atropine, which favors corneal penetration [20,55].

When considering the time needed by atropine to permeate through the tissues and to reach the steady state, longer lag times were observed for the cornea (1.8 h in water) in comparison with the sclera (0.76 h in water and 1.13 h in NaCl 0.9 %). These results are congruent with the structural differences of the tested tissues and the physicochemical properties of atropine mentioned above. The steady state fluxes (J) for the sclera were 33.29 μg/cm^{2}h for atropine solution in water and 29.16 μg/cm^{2}h for atropine solution in NaCl 0.9 %, and for the cornea 4.30 μg/cm^{2}h for atropine solution in water. The apparent permeability coefficients through sclera were 24x10^{-5} cm/s and 19x10^{-5} cm/s for solutions prepared in water or NaCl 0.9 %, respectively. The apparent permeability coefficient through cornea was 16x10^{-5} cm/s for atropine solution in water. Permeability coefficients were not calculated for the cornea treated with atropine solution in NaCl 0.9 % because quantitative

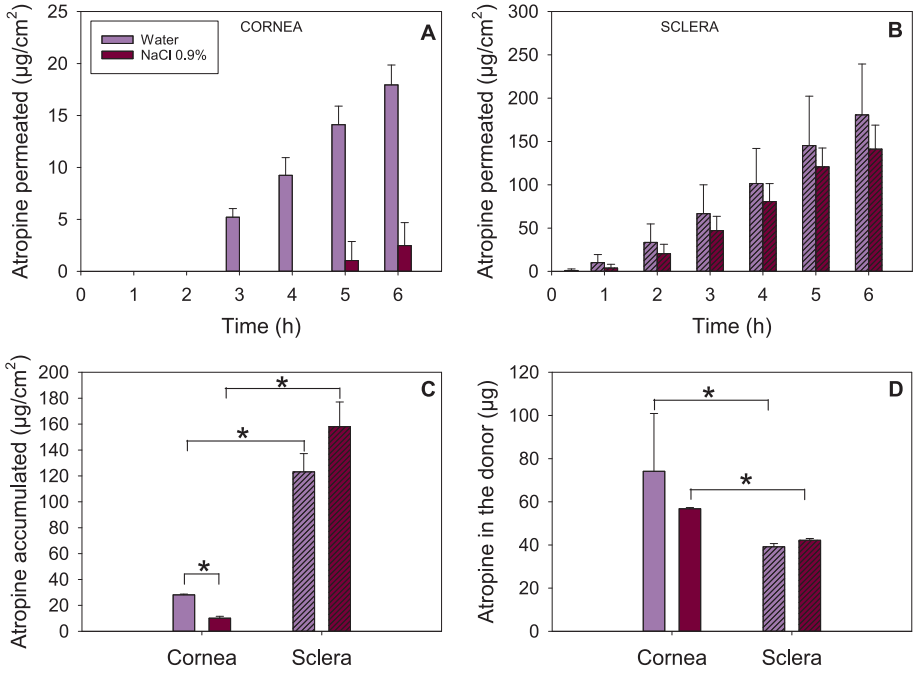

Fig. 3. Amounts of atropine permeated through (**A**) porcine cornea and (**B**) porcine sclera, (**C**) accumulated in tissues, and (**D**) remnant in the donor chamber after 6 h tests carried out with 1 mL of atropine solution (1 mg/mL) in water or NaCl 0.9 % medium. Mean values and standard deviations (n = 3). *Statistically significant differences, $p < 0.001$.

drug permeation values were obtained only in the last two sampling times, 5 and 6 h.

3.4. Atropine loading and release tests

During the loading and release tests, various parameters that can influence the interaction between atropine and the tested materials were systematically modified. The aim was to optimize the experimental protocol and gain deeper insight into the underlying processes.

While extending the time of soaking of the CLs in the drug solution from 24 h to 48 h was not seen to cause any enhance in the amount loaded, the preparation of atropine loading solution in water or NaCl 0.9 % played a crucial role. For hydrogels prepared with anionic monomers, the loading in water resulted in higher atropine uptake compared to the soaking in a NaCl 0.9 % solution, in which salt ions may shield the ionic interactions of atropine and the polymeric network ($p < 0.001$). This effect was not observed for the control formulation without anionic monomers (set coded with the number 1 after the name) and supplied CLs, except for ME (Fig. 4) also prepared with negatively charged co-monomers ($p < 0.001$).

The increase in the amounts of atropine loaded followed a concentration-dependent trend linked to the presence of the functional monomers MAA and AMPSA and was particularly marked with AMPSA because of its stronger acidity and higher percentage of ionization. The same behaviour was observed for ME, where the anionic monomers led to an increase in the amount of atropine loaded of 15-fold in water and 2-fold in NaCl 0.9 %. Generally, the hydrogels evidenced their interaction with atropine's positive charges at the working pH and thus, their composition-tunable capability to host atropine.

Release plots are shown in Fig. 5 **and S4**. Consistent with previous reports, all loading solutions prepared with non-buffered aqueous drug solutions exhibited pH values in the range of 5–6. Increasing drug concentration led to a slight pH decrease, although values always remained above 5. Distilled water typically has a pH close to 6, and the 0.1–0.2 mg/mL atropine solutions used for loading the CLs tested *in vivo* showed this same value, supporting good ocular compatibility. Although these values are slightly below the physiological pH range of tear fluid (7.4–7.7), the buffering capacity of tears provides tolerance over a broader interval. At pH 6, no stability concerns are anticipated for either the CL matrix or the drug [20,56,57].

After the loading, the release of atropine was evaluated in 6 mL of NaCl 0.9 % for all formulations (disregarding they were loaded in atropine solutions prepared in water or NaCl). As expected, all formulations containing anionic monomers and loaded in water were able to release higher amounts of atropine **(**Fig. 5 **and S4)**. However, no differences in the capacity to maintain a sustained release were observed for the same hydrogel or CL loaded in water or NaCl 0.9 %. This means that the salt content of the loading medium impacts on the amounts loaded but does not interfere with the release process.

Immediate release of atropine was observed from H-coded hydrogels as shown in Fig. 5**A**. This effect was more evident when the release was expressed as percentage, releasing nearly 100 % of the total amount loaded within the first hour **(Fig. S4A)**. Probably, this stems from a low affinity of the drug for the polymeric network. The same effect was observed for Esencia® CLs, except for ME CLs which showed a minor control of drug release during the first sampling times **(**Fig. 5**G and S4G)**. Differently, the incorporation of BzMA and MCS-MC12 in B and S formulations created hydrophobic regions capable of allocating the benzyl group and the carbonated substituents of atropine's tertiary amine resulting in a more controlled release **(**Fig. 5**B-F and S4B-F)** [38]. The addition of AMPSA led to the highest amounts of atropine released with the risk of exceeding the concentrations commonly used in the control of myopia progression [58]. Finally, Bx, Sb and Sx hydrogels were able to successfully extend the release of atropine for at least 6 h; the effect being more evident for Sx discs, which presented a higher proportion of BzMA and a lower proportion of MCS-MC12 in comparison with B and S formulations, respectively **(**Fig. 5**F and S4F)**.

In a next step, the loading and release profiles of B3x, S2x, EE and ME CLs were evaluated using two different soaking solutions of lower concentrations (0.1 and 0.2 mg/mL atropine in NaCl 0.9 %). In this case, the concentration of atropine in the loading solution was decreased from 1 mg/mL (in NaCl 0.9 % or water) to 0.1 and 0.2 mg/mL (in NaCl 0.8 % or 0.9 %). These concentrations were chosen to resemble as much as possible the usual dose of atropine in the control of myopia progression as eye drops in clinical practice and for safety reasons. The CLs were sterilized by autoclave (121 °C, 20 min), after placing them into vials containing 5 mL of atropine loading solution to satisfy the sterility requirements for commercially available CLs. The combination of the loading and sterilization processes in just one single-step would facilitate industrial scale-up. Atropine-loaded amounts for each composition are shown in **Fig. S5**. ME and EE CLs loaded slightly more atropine than B3x and S2x CLs in accordance with the results previously observed with a 1 mg/mL atropine loading solution. In all cases, the incorporation of atropine into the hydrogels when soaked in the diluted atropine solution (0.1 and 0.2 mg/mL) was lower than the observed in 1 mg/mL (Fig. 4). However, the affinity of the CLs for atropine was still quite high when soaked in the diluted drug solutions as indicated by the $K_{N/W}$ values, which ranged between 6.3 and 6.9 for B3x, S2x and EE CLs and increased up to 8.7 for ME CLs (Table 3). These $K_{N/W}$ values confirm that atropine effectively interacts with the polymer network rather than residing solely in the aqueous phase of the CLs.

In parallel, atropine solutions without CLs were also sterilized under identical conditions, and changes in their concentrations were monitored. After sterilization, the atropine concentration in the plain solution

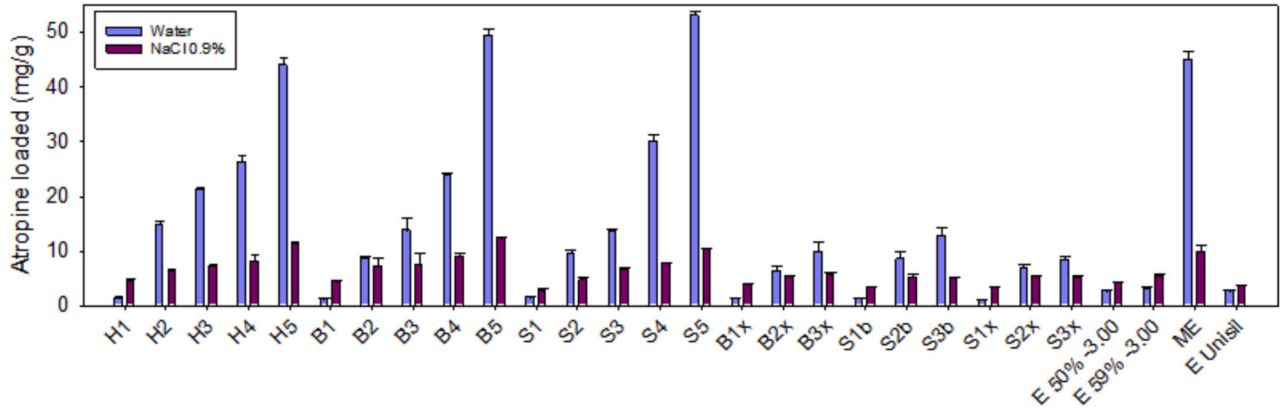

Fig. 4. Total amounts of atropine loaded into hydrogel discs and CLs after being immersed for 24 h in atropine solutions (1 mg/mL; 5 mL) prepared in water or NaCl 0.9 % medium. Mean values and standard deviations. Codes as in Tables 1 and 2.

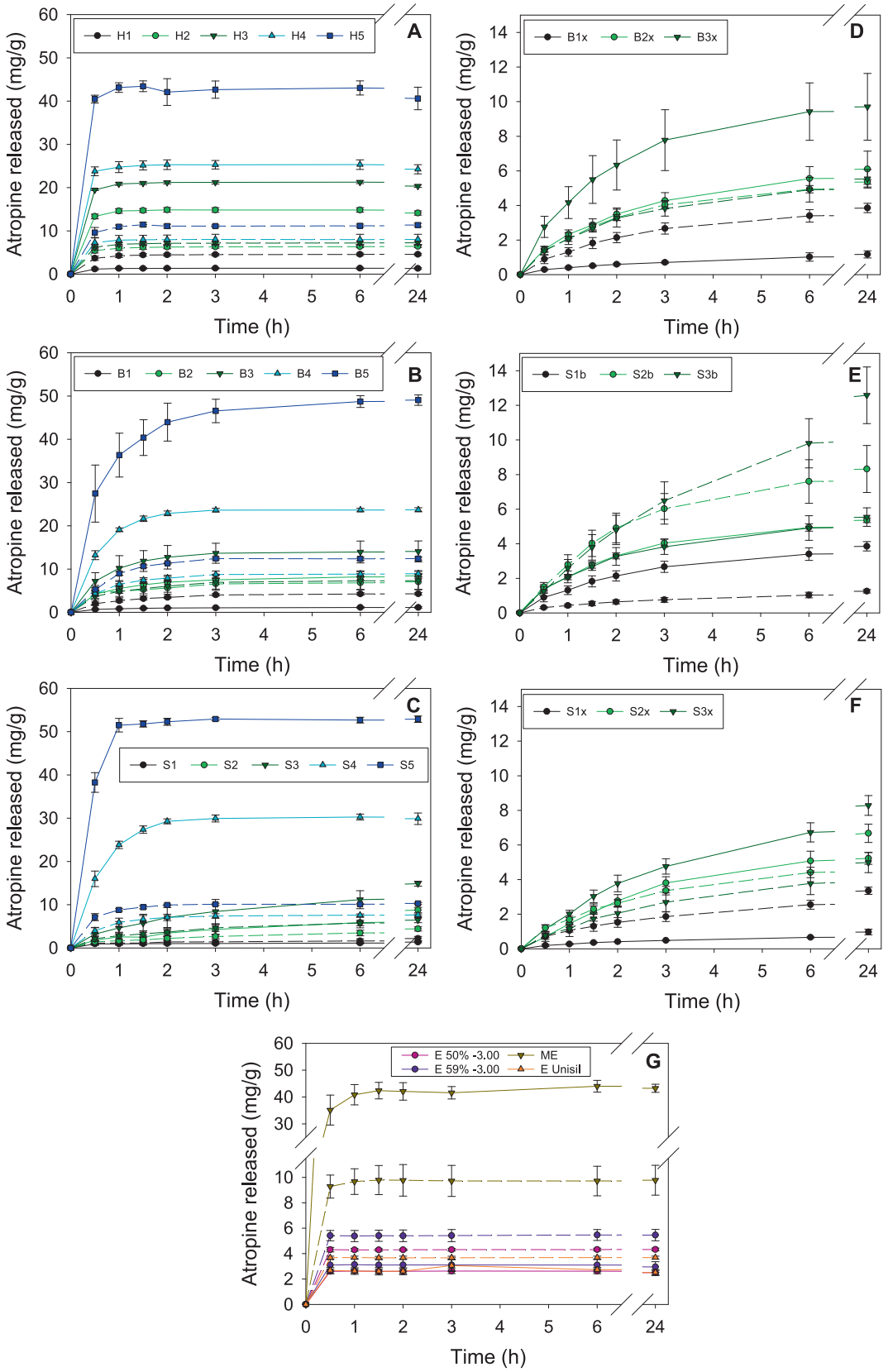

Fig. 5. Atropine release profiles (mg/g) from (**A**) H, (**B**) B, (**C**) S, (**D**) Bx, (**E**) Sb (**F**) Sx discs and (**G**) Esencia® CLs in 6 mL of NaCl 0.9 % at 36 °C and 180 rpm for 24 h. Solid and dotter lines refer to hydrogels soaked at room temperature and 180 rpm for 24 h in atropine (1 mg/mL; 5 mL) solution in water or NaCl 0.9 %, respectively. Mean values and standard deviations. Codes as in Tables 1 and 2.

Table 3
Atropine partition coefficient ($K_{N/W}$), area under the curve of concentration versus time (AUC), area under the curve of concentration x time versus time (AUMC), and mean residence time (MRT) of atropine in lachrymal fluid obtained for the 5th *in vivo* test.

Code	$K_{N/W}$	C_{max} (μg/mL)	t_{max} (h)	$AUC_{0\text{-}6h}$ (μg/mL·h)	$AUMC_{0\text{-}6h}$ (μg·h/mL·h)	MRT (h)
B3x CL	6.87	40.21 ± 34.29	0.083	32.31 ± 20.46	26.28 ± 17.45	0.79 ± 0.18
S2x CL	6.29	19.53 ± 3.79	0.083	31.66 ± 13.85	47.27 ± 23.51	1.46 ± 0.09
EE CL	6.91	42.06 ± 6.17	0.083	42.58 ± 12.02	41.73 ± 21.33	0.94 ± 0.19
ME CL	8.68	36.24 ± 7.60	0.25	45.39 ± 3.36	38.21 ± 7.30	0.84 ± 0.17
Eye drops	–	26.89 ± 36.04	0.083	6.07 ± 8.93	2.76 ± 3.44	0.73 ± 0.41

(without CLs) decreased by approximately 50 % (from 100 μg/mL down to ~ 52 μg/mL) likely due to ester hydrolysis [59]. In contrast, vials containing CLs exhibited a different behavior: the atropine concentration decreased due to the loading, but in all cases was above 80 μg/mL, suggesting a protective effect of the hydrogel matrix against degradation. Indeed, negatively charged hydrogels have been reported to slow down ester hydrolysis [60], which could explain the enhanced stability of atropine when loaded into the chosen CLs.

The release of all atropine-loaded CLs was carried out in 6 mL of NaCl 0.9 % as previously described (36 °C, 180 rpm, 24 h). S2x and B3x CLs were able to provide more controlled release profiles than Esencia®, sustaining the release for at least 4 h **(**Fig. 6 **and S6)**. No statistical differences were observed in the maximum amounts of atropine released when the concentration of the loading solution was doubled, something expected considering the amounts loaded.

All developed CLs released 15–25 μg of atropine (Fig. 6**B**). This is very similar to the amounts observed for other commercial CLs loaded with a 10 times higher concentration solution. Our findings suggest that exposing the lenses to such high drug-loading concentrations is not recommended, as it may lead to serious ocular side effects and, if accidentally ingested, systemic toxicity [23]. Also relevantly, the accumulative amounts of atropine released and the release rate observed (Fig. 6) were similar to those previously reported for silicone CLs containing an implant with atropine embedded during polymerization [26]. However, our protocol offers the advantage that the CLs can be fabricated using standard polymerization techniques and then loaded afterward. This allows the drug-loading and sterilization steps to occur simultaneously within a single process, thereby streamlining industrial scale-up. Finally, considering the current clinical practice of atropine for myopia control, where a single drop (50 μL) of a 0.1 mg/mL atropine solution is instilled per day, i.e. 5 μg dose, and assuming a common CL weight of 14 mg, our developed formulations B3x and S2x would ensure therapeutically relevant amounts of atropine in the lachrymal fluid (Fig. 6**B**).

3.5. *HET-CAM test*

The HET-CAM test was performed here for a preliminary screening of the ocular compatibility of the atropine-loaded formulations. The HET-CAM assay relies on the similarity between the vascularized CAM of

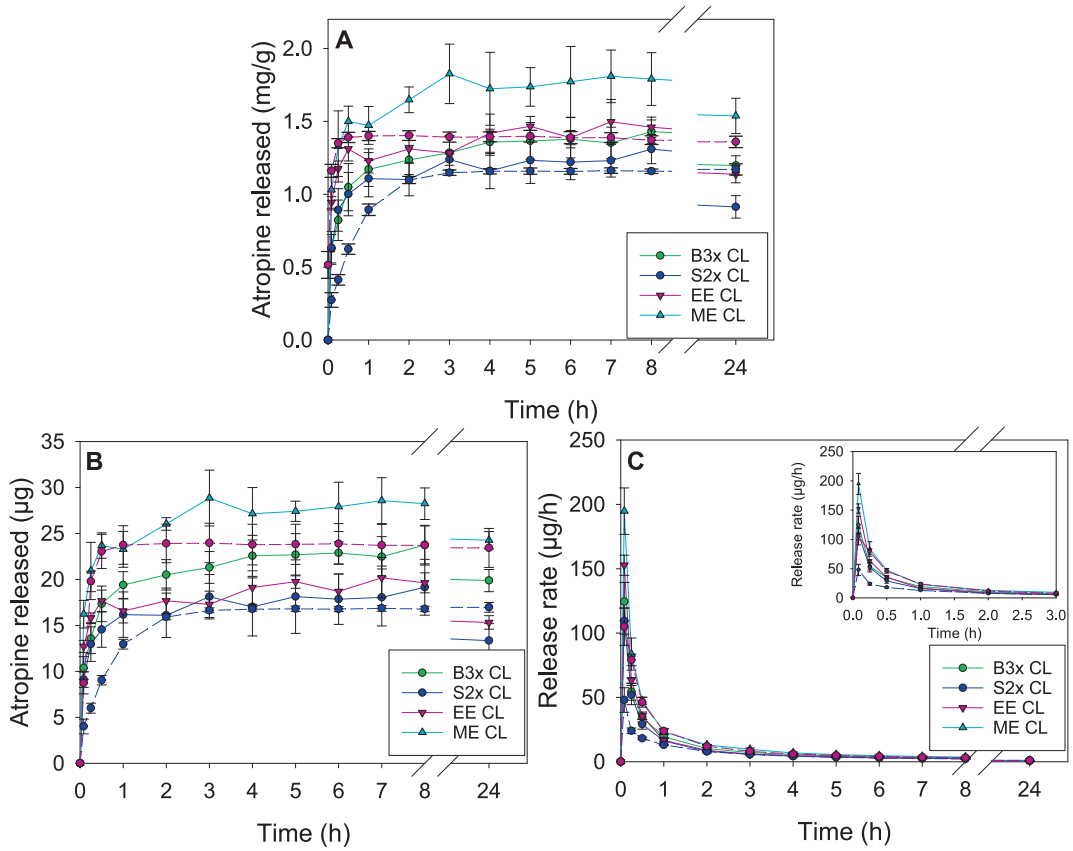

Fig. 6. Atropine release profiles *in vitro* from all sterilized CLs expressed as (**A**) cumulative drug released per mass of CL, (**B**) cumulative drug released per CL unit, and (**C**) release rate as a function of time (μg/h) after being loaded in 5 mL of 0.1 (solid lines) or 0.2 (dotted lines) mg/mL of atropine in NaCl 0.9 % medium at room temperature and 180 rpm. Mean values and standard deviations. Codes as in Tables 1 and 2.

fertilized chicken egg embryos and the vascularized mucosal ocular tissues, the potential irritant response against a product is similar to the one induced on the ocular conjunctiva [61]. Synthesized H hydrogel discs, commercial ME and EE CLs, and developed B3x and S2x CLs successfully passed the HET-CAM test as long as no lysis, hemorrhage and coagulation were observed after 5-min of exposure (**Figs. S7 and S8**). The IS of the positive controls (NaOH 0.1 N) were 19.24 and 19.52 in separate trials, while the negative control (NaCl 0.9 %) showed an IS of 0.0 in all cases.

3.6. *In vivo* experiments: Atropine release and ocular biodistribution

After successfully completing all prior experiments, the ability of S2x, B3x, EE, and ME CLs to deliver therapeutically relevant amounts of atropine to the tear fluid and facilitate its penetration into various ocular tissues was evaluated through five independent *in vivo* studies. New Zealand White rabbits were selected as the experimental animal model due to their widespread use in ophthalmic research and the anatomical and physiological similarities between rabbit and human eyes [62,63]. Prior to the *in vivo* study, the oxygen permeability of the atropine-loaded CLs was confirmed using the polarographic method, in accordance with ISO 18369–4:2017 [64]. Oxygen diffusion through the lens was measured with a Model 201 T O_2 permeometer (Createch, Albany, CA, USA) equipped with a polarographic cell consisting of a gold cathode and a silver anode in a chamber maintained at controlled temperature (25 °C) and high humidity. A drop of borax buffer was applied to the cell before placing the CL, and the current intensity was recorded until a steady state was reached. Oxygen permeability was then calculated from the current intensity values as previously described [64,65]. The mean oxygen permeability values for S2x, B3x, EE, and ME CLs were 21.9, 25.2, 49.6, and 39.2 barrers, respectively, with standard deviations below 10 % [64], failing within the typical range for soft CLs.

The slit-lamp images of the ocular surface recorded for all rabbits before the beginning of the experiments, at 1 h, and at the end (6 or 8 h) confirmed the good tolerance of all formulations, since no ocular damage was induced (**Fig. S9 and S10**). The Draize irritation criteria and the

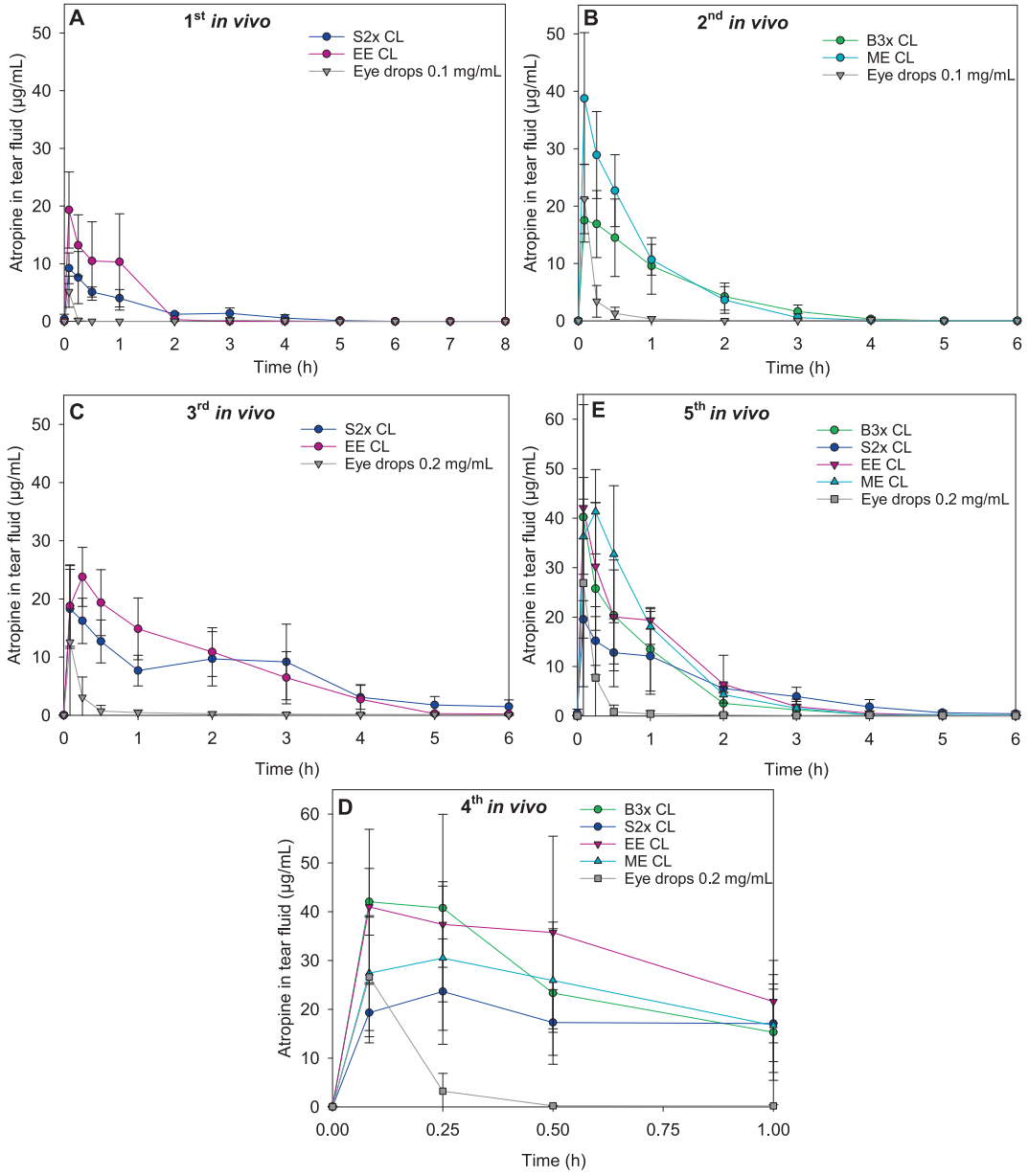

Fig. 7. *In vivo* tear fluid levels of atropine recorded after instillation of one single drop (50 µL) of (**A, B**) 0.1 mg/mL or (**C**) 0.2 mg/mL atropine in NaCl 0.9 % or (**D, E**) NaCl 0.8 % solution and during wearing of CLs for (**A**) 8 h, (**B, C, E**) 6 h and (**D**) 1 h. Mean values and standard deviations (n = 4). Codes as in Tables 1 and 2.

scores obtained for all animals are shown in **Table S2**. The slight irritation score observed for some of the rabbits 1 h after the beginning of the experiment could be due to the increased number of Schirmer tests performed just before. Similarly, the slight irritation observed in some cases at the end of the experiment could be attributed to the fact that the CLs did not fit the rabbit's eye perfectly. As expected, the pupilar diameter of the right eye (**Table S3**) increased approx. 1 mm after administration due to the mydriatic effect of atropine [20]. This effect, although small, was more evident at 1 h, when the animals were exposed to the light of the slit-lamp to take photos.

3.6.1. *Atropine levels in tear fluid*

The *in vivo* release profiles of atropine in tear fluid are shown in Fig. 7 for all experiments. In the first *in vivo* experiment, the rabbits were euthanized after 8 h. However, as no atropine was quantified in tear fluid 5 h post-instillation or CL insertion, the duration of the next *in vivo* experiments was shortened to 1 and 6 h. For each *in vivo* test, the CLs were loaded by soaking them in the atropine eye drop formulation used in the parallel test, with the same concentration and medium. Surprisingly, in contrast to previous works carried out for other drug-loaded CLs [29,31], the concentration of atropine in tear fluid when delivered as eye drops did not exceed the level provided by the CLs in almost any case. This finding is clearly related to the fact that one eye drop delivers 5 or 10 μg atropine (for 0.1 and 0.2 mg/mL solution, respectively) while atropine dose in the CLs ranged between 15 and 25 μg (Fig. 6**B**) and highlights the affinity of the selected biomaterials for the drug. The higher concentrations of atropine observed for CLs in tear fluid could also be explained by a rapid diffusion of atropine to the post-lens film, which would increase its residence time on the ocular surface and thereby slow down tear drug clearance [66].

In general, the highest concentration of atropine in the rabbit tear fluid was observed 5 min post-administration of the assayed formulations or when it was not the case, 15 min after the placement of the CLs. No relevant differences after doubling the loading concentration for S2x (9.21 ± 2.67 μg/mL vs 18.29 ± 6.79 μg/mL), EE (19.31 ± 6.59 μg/mL vs 18.79 ± 6.94 μg/mL) and the eye drops (5.15 ± 2.70 μg/mL and 21.24 ± 6.04 μg/mL vs 12.56 ± 13.31 μg/mL) were observed. B3x and ME CLs loaded in a 0.1 mg/mL atropine in NaCl 0.9 % solution led to 17.50 ± 3.77 μg/mL and 38.75 ± 11.48 μg/mL of atropine in tear fluid after 5 min of administration, respectively.

The two *in vivo* tests performed with CLs loaded in 0.2 mg/mL atropine in NaCl 0.8 % (4th and 5th *in vivo* experiment; Fig. 7 **D and E**) provided peak concentrations of atropine in tear fluid of 42.02 ± 6.84 μg/mL and 40.21 ± 34.29 μg/mL for B3x, 19.30 ± 6.17 μg/mL and 19.53 ± 3.79 μg/mL for S2x, 41.00 ± 15.88 μg/mL and 42.06 ± 6.17 μg/mL for EE, 27.42 ± 11.78 μg/mL and 36.24 ± 7.60 μg/mL for ME, and 26.63 ± 12.26 μg/mL and 26.89 ± 36.04 μg/mL for eye drops, in independent experiments lasting 1 h and 6 h respectively. The minimal dispersion in mean atropine concentrations between the two *in vivo* studies demonstrates the reliability and consistency of the atropine-loaded CLs.

Following the first sampling point, atropine concentrations in the tear fluid of the eye drop groups declined rapidly, becoming nearly undetectable within 1 h of the beginning of the experiment. In contrast, although a gradual decline was also observed for all CLs groups, S2x and B3x lenses sustained atropine release *in vivo* for at least 4 h, consistent with previously obtained *in vitro* results. Esencia® CLs showed atropine release profiles similar to the developed CLs, but atropine levels in tear fluid decayed more rapidly. The sustained release observed *in vivo* for EE CLs, unlike the immediate release seen *in vitro,* may be attributed to the lower volume of tear fluid available *in vivo* compared to the 6 mL of the NaCl 0.9 % release medium used *in vitro* [22]. Although doubling the atropine concentration did not raise peak levels, it led to higher and more sustained atropine concentrations in the tear fluid over time. Atropine concentrations in the tear fluid of the left (untreated) eyes were not quantifiable in any group.

The pharmacokinetic parameters obtained from the atropine concentration profiles in tear fluid for the CLs (15–25 μg) and eye drops (5 μg dose) tested in the 5th *in vivo* test are compiled in Table 3. The AUC was remarkably lower for the eye drops, even when the values were normalized by the doses. Considering that the CLs loaded 3 times (S2x and EE) and 4 times (B3x and ME) the dose of atropine contained in one drop, the AUC values were 5 times (S2x and B3x) and 7 times (EE and ME) higher than those recorded for the eye drops. This means that the CLs provided a nearly two times higher AUC. This result is in good agreement with previous reports on CLs loaded by soaking with pravastatin [31] or betaxolol hydrochloride [67]. Interestingly, despite the CLs notably extended the permanence of atropine in the lachrymal fluid (as observed more clearly in Fig. 7**D**) a significant increase in the MRT was only recorded for S2x CLs, which duplicated the value recorded for the eye drops.

Levy plots (Fig. 8) were used to assess *in vitro-in vivo* (IVIV) correlations. The *in vivo* release profiles (Fig. 8**A**), derived using Eq. (2), demonstrated sustained atropine delivery for up to 4 h, with the effect being most pronounced for the S2x CLs, which exhibited statistically significant differences at 0.5, 1, 2, 3, and 4 h. Interestingly, drug release appeared to be more prolonged *in vivo* (Fig. 8**A**) compared to *in vitro* conditions (e.g., Fig. 6**B**). This may be attributed to the smaller available tear volume *in vivo* (a few microliters versus 6 mL *in vitro*), as well as the presence of proteins in the tear fluid, which may adsorb onto the CL surface. In general, CLs prepared with MAA are known to be rapidly coated with a lysozyme layer upon ocular insertion [44], which can hinder drug release.

This slower initial release rate *in vivo* was clearly reflected in the deviation of the Levy plots from the origin (Fig. 8**B and 8C**). For better visualization of early-time deviations, the regression lines were not constrained to pass through the origin. Notably, the initial burst observed *in vitro* was absent *in vivo*. Similar trends of lower initial *in vivo* release compared to *in vitro* have also been reported for CLs loaded with betaxolol hydrochloride [67].

The correlation coefficient (r^2) values obtained for the developed B3x and S2x CLs were closer to 1 than those for commercially available EE and ME CLs, indicating stronger IVIV correlations.

The slopes of the Levy plots for B3x and S2x CLs were 2.01 (standard error: 0.16) and 1.24 (standard error: 0.13), respectively. In comparison, slopes for EE and ME CLs were 3.26 (standard error: 0.79) and 2.81 (standard error: 0.36), respectively. These results suggest strong correlations with positive deviations. Slopes greater than 1 indicate that once release is initiated *in vivo*, it proceeds relatively rapidly. Similar positive deviations (e.g., 1.54 ± 0.07) have been reported for pravastatin-loaded CLs [31], particularly when the *in vitro* release medium lacked proteins. The absence of standardized methodologies for evaluating drug release from CLs and other solid or semisolid ocular drug delivery systems remains a significant limitation for predicting *in vivo* performance and comparing findings across studies [22].

3.6.2. *Atropine levels in ocular tissues*

As mentioned above, *in vivo* experiments of different durations (1, 6 and 8 h) were conducted. The shortest assay (1 h) was designed to assess the initial distribution of atropine during the early phase following instillation. On one hand, higher atropine levels might be detected in various ocular tissues due to limited physiological clearance at this stage. On the other hand, lower levels could also be observed, depending on the time required for the drug to reach each tissue.

Previous studies on the distribution of atropine in the ocular tissues after topical administration in rabbits have shown that atropine can accumulate in the cornea and reach the posterior segment of the eye [53,68]. However, the number of studies reporting on atropine levels in tissues after ocular administration (rabbits) is still low, and most of them are based on the administration of atropine as eye drops. Here, the capacity of atropine released from different medicated CLs to permeate through and accumulate in the anterior and posterior ocular tissues was

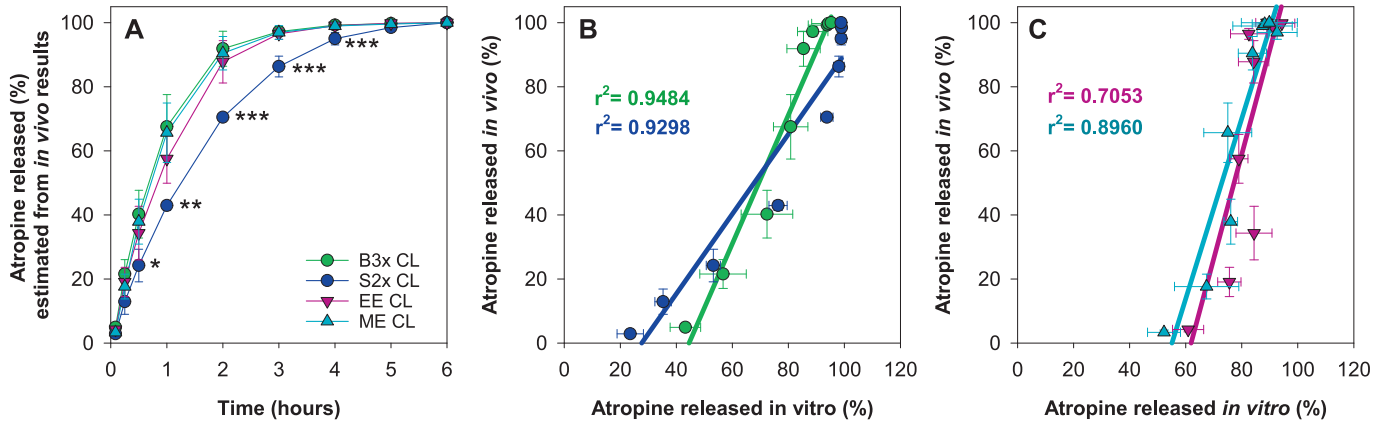

Fig. 8. (A) Atropine release profiles *in vivo* and (B,C) Levy plots for *in vivo* vs. *in vitro* percentage of drug released. The *in vitro* tests data were performed in 6 mL of NaCl 0.9 %. *Statistically significant difference of S2x CL compared with EE CL and ME CL ($p < 0.05$). ** and *** indicate statistically significant differences of S2x CL compared with all other formulations at $p < 0.01$ and $p < 0.001$, respectively.

evaluated and compared against eye drops with the same atropine concentration as the CLs loading solution. Both treated and untreated eyes of all rabbits were analyzed, and atropine was detected to a greater or lesser extent in all tissues (aqueous humor, cornea, vitreous humor, retina and sclera) after 1, 6 and 8 h treatment with eye drops and CLs, as depicted in Fig. 9. Crystalline lens samples could not be analyzed due to overpressure column in the UPLC-MS equipment.

In the longest *in vivo* tests (6 and 8 h) results showed a preferential atropine distribution in the cornea, retina and sclera over the aqueous and vitreous humors. This could be explained by the physicochemical properties of atropine sulfate, such as the ionized state and the high molecular weight, which may hinder its ability to permeate through the corneal tissue and determine its preference for other than transcorneal and transvitreal pathways. However, the transcorneal route cannot be discarded considering the atropine partition coefficient (logP = 1.83). In line with this assumption, in the 1-hour experiment, the highest atropine amounts were found in the aqueous humor and the cornea of the treated eye, followed by the retina and sclera tissues.

The ability of the studied CLs to promote the accumulation of atropine in the retina and sclera is noteworthy. Although the exact mechanism responsible for myopia progression is yet to be established, the retina and the sclera/choroid seem to be the main tissues involved in this process. The amounts registered in aqueous humor, cornea, vitreous humor and sclera (**Table S4**) after 6 h treatment in the eye drops group were in good agreement with the amounts previously reported for a 0.1 mg/mL atropine solution [68]. The higher amounts of atropine recorded in all tissues after 6 h of wearing of B3x and ME CLs **(**Fig. 9**C and 9D)** could be due to the higher and more sustained release of atropine as observed in the tear fluid levels registered. In general, no statistically significant differences were observed in the amounts of atropine accumulated between different formulations for the same ocular tissue when loaded by soaking in 0.1 mg/mL atropine solution. Differently, the amounts registered notably increased in the cornea, the vitreous humor, the retina and the sclera after doubling the concentration of the loading solution **(**Fig. 9**E and 9F)**. This increase in tissue levels agreed well with a previous report showing higher levels of atropine in the ocular tissues after the instillation of a concentrated atropine solution (10 mg/mL) compared to a diluted eye drop (0.1 mg/mL) [68]. Moreover, after doubling the soaking concentration, the capacity of S2x and EE CLs to provide higher amounts of atropine to the ocular tissues compared to the eye drops was more evident, especially for retina and sclera tissues.

Apart from doubling the concentration, in a parallel study, the duration of the assay was reduced from 6 h to 1 h. In this situation, the presence of atropine was lower in all tissues (cornea, vitreous humor, retina and sclera) except in the aqueous humor in which quantifiable amounts were found (20.58 ± 13.72 μg/mL for B3x, 23.12 ± 19.67 μg/mL for S2x, 5.57 ± 1.85 μg/mL for EE, 6.91 ± 2.50 μg/mL for ME and 4.48 ± 4.79 and μg/mL for 0.2 mg/mL eye drops) (**Table S4**), thus suggesting the distribution equilibrium between tissues had not been reached yet.

The amounts registered for all formulations in the 1st and 2nd *in vivo* experiments were slightly lower than the amounts previously reported after 6 h administration of an atropine-loaded film [68], but this was solved in the 3rd experiment by doubling the concentration of atropine soaking solution (from 0.1 to 0.2 mg/mL) where the amounts registered were notably higher. This suggests that by modifying the concentration of atropine in the eye drops or the CLs loading solution it is possible to increase the amounts of atropine in the ocular tissues.

Regarding the loading solution, in the last two *in vivo* experiments (4th and 5th) the concentration of NaCl was decreased from 0.9 % to 0.8 % to obtain some level of hypotonicity that could enhance the penetration of the drug into the ocular tissues [36,37]. In this case, this variation did not appear to provide an advantage in terms of permeation, but it deserves further analysis.

Atropine in the left eyes (without treatment) was also analyzed for all formulations **(**Fig. 9**B, 9D, 9F, 9H and 9 J)**, and lower levels were quantified than in the treated eyes, but confirms the capacity of atropine to pass into the other eye. Wang et al. [54] observed a similar phenomenon, although more marked, after 5 h instillation of 50 μL of 10 mg/mL atropine solution and postulated that this crossover effect was due to systemic absorption pathways. In the one-hour experiment, amounts of atropine were quantified in the left eye (with some variability), thus suggesting that this effect may already have been initiated at that time. The preferential distribution of atropine in the cornea, retina and sclera of untreated eyes was the same as in the contralateral eyes, but to a lesser extent (**Table S4**).

In terms of absolute quantities, the developed CLs have demonstrated the capacity to release at least the doses used in clinical practice. Among the dissected ocular tissues, the retina and sclera have been most strongly associated with the progression of myopia. Although the literature on this topic is limited, Wang et al. [54] reported atropine concentrations of approximately 400 ± 90 ng/g in the retina, 4350 ± 2150 ng/g in the anterior sclera, 2420 ± 1190 ng/g in the equatorial sclera and 779 ± 290 ng/g in the posterior sclera after instilling 50 μL of a 10 mg/mL (1 %) atropine sulfate solution. In our study, similar levels were achieved when S2x and EE CLs were loaded by soaking in a 0.2 mg/mL atropine solution in 0.9 % NaCl. Notably, even 0.1 mg/mL (0.01 %) atropine eye drops have been shown to effectively control myopia progression, suggesting that lower concentrations of atropine in ocular tissues may still hold therapeutic relevance [7].

Overall, the *in vivo* studies provided a more accurate understanding of the actual drug delivery performance of the most promising

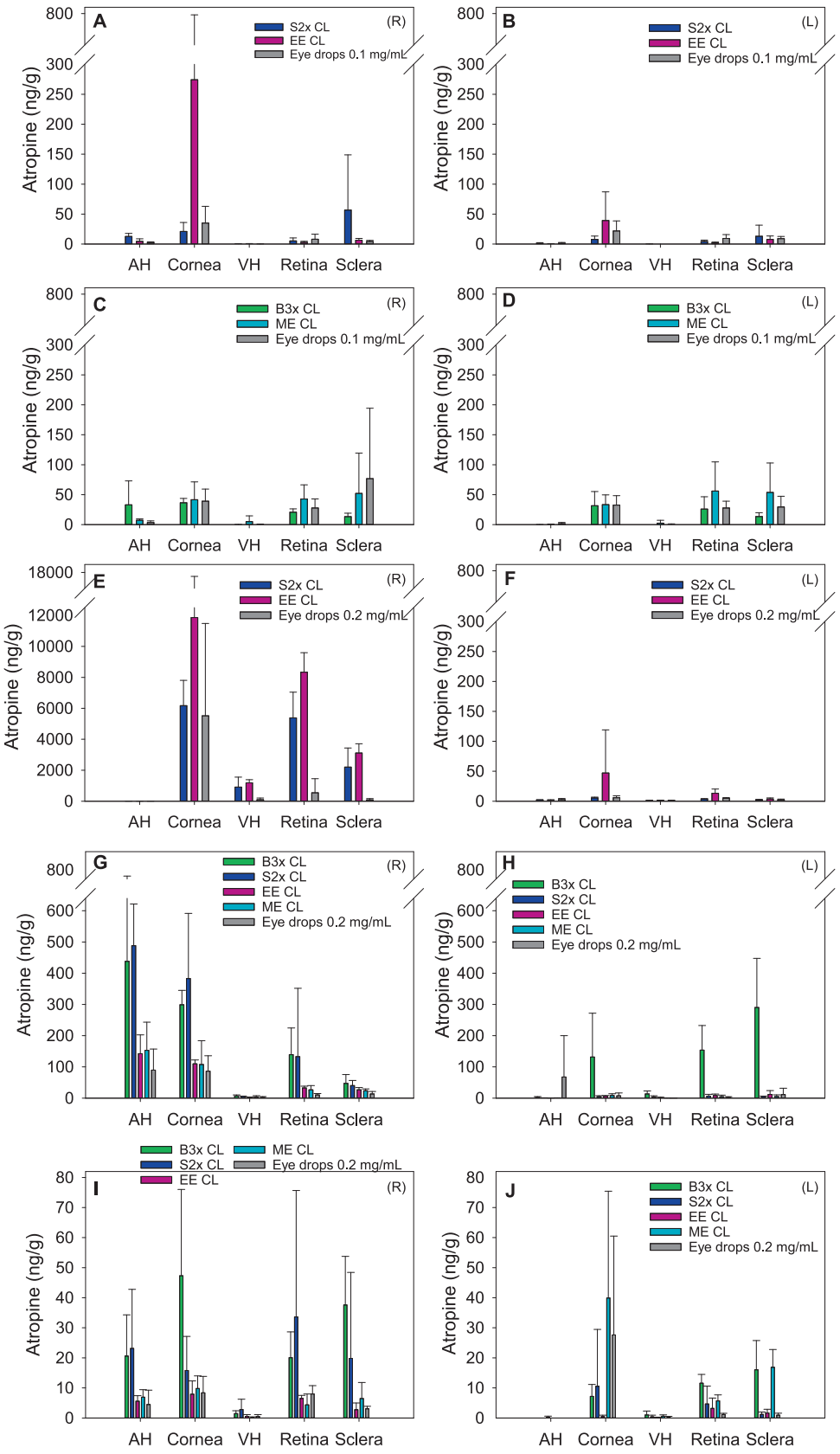

Fig. 9. Levels of atropine in ocular tissues (aqueous humor, cornea, vitreous humor, retina, and sclera) after (**A, B**) 8 h, (**C, D, E, F, I, J**) 6 h and (**G, H**) 1 h of wearing of (**A, B, C, D**) atropine-loaded CLs loaded in 0.1 mg/mL and (**E, F, G, H, I, J**) 0.2 mg/mL atropine solutions or instillation of one single drop (50 μL) of (**A, B, C, D**) 0.1 mg/mL and (**E, F, G, H, I, J**) 0.2 mg/mL atropine in (**E, F**) NaCl 0.9 % or (**G, H, I, J**) NaCl 0.8 % solution for the (**R**) right and (**L**) left eyes. The bars represent mean values and standard deviations (n = 4).

formulations tested *in vitro*. The observed drug distribution profiles across ocular structures helped clarify the preferred routes of atropine diffusion and its accumulation in tissues that had not been clearly characterized before. These *in vivo* studies enabled quantitative assessment of atropine's ocular bioavailability and biodistribution, which is important not only for understanding its pharmacokinetics but also for evaluating whether the measured tissue concentrations are therapeutically meaningful. Moreover, the data generated could help in the future to establish correlations with *in vitro* and pharmacokinetic models, to progressively reduce the number of animals.

4. Conclusions

Tailored HEMA- and silicone-based CLs capable of loading and sustainably releasing therapeutically relevant amounts of atropine were successfully developed. All formulations met key requirements for commercialization, including solvent uptake, light transmission, mechanical integrity and ocular compatibility. The developed CLs can be used as neutral lenses or further tailored to incorporate optical properties beneficial for myopia management. Atropine loading was more efficient in water than in NaCl, particularly for CLs incorporating ionic monomers, underscoring the role of ionic interactions in drug binding. Copolymerization of anionic and hydrophobic monomers in various ratios enabled sustained drug release.

Ex vivo permeability studies revealed that atropine permeates the sclera more rapidly and extensively than the cornea. The most promising formulations (B3x, S2x, EE, and ME) were successfully loaded and sterilized via steam heat in a single step, without compromising their capacity to load or release atropine. *In vivo* results confirmed the biocompatibility of the CLs and their ability to provide sustained atropine release into the tear fluid for at least four hours, especially after soaking in a 0.2 mg/mL atropine solution. Quantification of atropine in ocular tissues yielded valuable insight into its distribution, with therapeutically relevant concentrations detected in the cornea and sclera. Atropine was also found in the posterior segment of the eye, which is an important observation given the role of the retina and sclera in myopia progression.

While our study provides encouraging results, several limitations should be acknowledged. First, we tested the intervention only in visually healthy animals; therefore, its preventive or therapeutic efficacy against myopia could not be assessed. Moreover, commonly used animal models may not fully replicate the progression of myopia in humans, highlighting the need for future pilot trials in patients. In addition, the long-term stability of the drug within the lens matrix should be examined under standardized conditions to establish the shelf-life of the medicated contact lenses.

Despite these limitations, our findings support the feasibility of developing hydrogel contact lenses that engage in both ionic and hydrophobic interactions with atropine, thereby offering a dual mechanism for effective drug delivery. Such drug–lens combination products represent a promising strategy for controlling myopia progression.

CRediT authorship contribution statement

Inés García-del-Valle: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Maria Vivero-Lopez:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Angel Concheiro:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Carmen Alvarez-Lorenzo:** Writing – review & editing, Validation, Supervision, Software, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

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. They are coinventors of Patent WO2024156642A1 Hydrogels containing atropine.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eurpolymj.2025.114293.

Data availability

Data will be made available on request.

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