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Ocular biocompatibility of novel Cyclosporin A formulations based on methoxy poly(ethylene glycol)-hexylsubstituted poly(lactide) micelle carriers

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ABSTRACT

Topical ocular drug delivery has always been a challenge for pharmaceutical technology scientists. In the last two decades, many nano-systems have been studied to find ways to overcome the typical problems of topical ocular therapy, such as difficult corneal penetration and poor drug availability. In this study, methoxy poly(ethylene glycol)-hexylsubstituted poly(lactides) (MPEG-hexPLA) micelle formulations, which are promising nanocarriers for poorly water soluble drugs, were investigated for the delivery of Cyclosporin A (CsA) to the eye. As a new possible pharmaceutical excipient, the ocular compatibility of MPEG-hexPLA micelle formulations was evaluated. An *in vitro* biocompatibility assessment on human corneal epithelial cells was carried out using different tests. Cytotoxicity was studied by using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT), and clonogenic tests and revealed that the CsA formulations and copolymer solutions were not toxic. After incubation with MPEG-hexPLA micelle formulations, the activation of caspase-dependent and -independent apoptosis as well as autophagy was evaluated using immunohistochemistry by analyzing the localization of four antibodies: (1) anti-caspase 3; (2) anti-apoptotic inducing factor (AIF); (3) anti-IL-Dnase II and (4) anti-microtubule-associated protein 1 light chain 3 (LC3). No apoptosis was induced when the cells were treated with the micelle solutions that were either unloaded or loaded with CsA. The ocular tolerance was assessed *in vivo* on rabbit eyes by Confocal Laser Scanning Ophthalmoscopy (CLSO), and very good tolerability was seen. The observed corneal surface was comparable to a control surface that was treated with a 0.9% NaCl solution. In conclusion, these results demonstrate that MPEG-hexPLA micelles are promising drug carriers for ocular diseases involving the activation of cytokines, such as dry eye syndrome and autoimmune uveitis, or for the prevention of corneal graft rejection.

1. Introduction

In the last decade, many colloidal systems (Sahoo et al., 2008) and polymeric sustained delivery systems (Bourges et al., 2006) have been developed for ophthalmic applications, such as nanosuspensions (Pignatello et al., 2002), liposomes (Shen and Tu, 2007), or nanocapsules (De Campos et al., 2003). A major issue in the field is poor drug bioavailability due to a short precorneal residence time and poor corneal penetration (Urtti, 2006). Another problem is the increased occurrence of side effects when administering a drug systemically or intraocularly to reach therapeutic levels (Sahoo et al., 2008; del Amo and Urtti, 2008).

Concerning the topical ocular application, liposomes have shown promising results in enhancing the corneal penetration of penicillin G and indoxole in *ex vivo* and *in vivo* studies, respectively (Schaeffer and Krohn, 1982). Liposomes also increased the ocular bioavailability of ganciclovir in rabbit eyes (Shen and Tu, 2007).

Sparfloxacin-loaded PLGA nanoparticles showed prolonged retention at the corneal site, as revealed by gamma-scintigraphy (Gupta et al., 2010). However, no nanoparticulate product has yet reached the market. New commercial formulations are mostly emulsions or nanoemulsions, such as Restasis® or Cyclokat® for the treatment of dry eye syndrome, the latter formulations are on

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the market in the USA and in clinical phase III trials in Europe, respectively.

Cyclosporin A (CsA) is a potent immunosuppressant that is widely used to prevent corneal graft rejection and to treat autoimmune uveitis and dry eye syndrome (Kulkarni, 2001; Italia et al., 2006). However, due to its very poor water solubility, the formulation of CsA is a challenge (Lallemand et al., 2003). Its solubilization within nanocarriers, such as hyaluronic acid coated poly-3-caprolactone nanospheres, showed promising results for increased corneal penetration in *in vivo* studies (Yenice et al., 2008).

Nanoparticulate systems must be evaluated not only in terms of efficacy, but also in terms of biocompatibility. Their toxicity must be adequately determined both *in vitro* and *in vivo*. Many different tests are available to assess the toxicity of nanoparticulate systems (Jones and Grainger, 2009; Aillon et al., 2009). The *in vitro* toxicity is often tested on cells. The choice of the cellular model is extremely important because the selected cell line must reproduce a response to the material that is similar to what occurs *in vivo* (Jones and Grainger, 2009). A large series of cytotoxicity tests for different cellular mechanisms is available (Mannerstrom et al., 2002; Haritoglou et al., 2005; Lewinski et al., 2008). To evaluate the ocular tolerance of topically applied formulations *in vivo,* the Draize test is classically used (Draize et al., 1944). This test allows for the assessment of acute, intermediate and chronic exposures of the tested compounds. The most important drawbacks of this test are the subjectivity of the evaluation of corneal lesions and the low reproducibility (Wilhelmus, 2001). Recently, a large amount of effort has been put into developing alternative methods to the Draize eye test (Kurishita et al., 1999; Balls et al., 1999; Hartung et al., 2010). Furrer et al. (2000) developed an alternative *in vivo* method based on Confocal Laser Scanning Ophthalmoscopy (CLSO) for the evaluation of the possible corneal effects of repeated applied formulations. This test gives objective numerical responses and is more reproducible than the Draize test, it is a good tool to assess the ocular safety of new ophthalmic formulations and was therefore applied in this current study.

Recently, our group prepared polymeric micelles based on PEG-hexylsubstituted poly(lactides) (PEG-hexPLA) block copolymers (Trimaille et al., 2006). These micelles showed a high drug loading capacity for Cyclosporin A and were stable in aqueous formulations (Mondon et al., 2010). Furthermore, the feasibility of lyophilizing MPEG-hexPLA micelle solutions to increase the stability of the formulation has been demonstrated (Di Tommaso et al., 2010). In particular, these polymeric micelles have promising characteristics that may be applied to ophthalmic applications. For example, their nanosize leads to transparent solutions and may facilitate good corneal penetration, which will increase the bioavailability of the incorporated drug. However, before assessing the corneal penetration and pharmacokinetics, it is necessary to test the ocular biocompatibility of this system because any disruption of corneal tissues due to toxicity from the carrier would lead to misleading results. Because of this fact, the aim of this work was to develop a well-characterized CsA/MPEG-hexPLA micelle formulation and to evaluate its *in vitro* and *in vivo* ocular biocompatibility as a novel carrier for the topical delivery of CsA to the eye.

2. Materials and methods

2.1. Materials

α-Hydroxyoctanoic acid and 3,6-dihexyl-1,4-dioxane-2,5-dione (dihexylsubstituted lactide, hexLA) were synthesized as described previously (Trimaille et al., 2004). Methoxy poly(ethylene glycol) (2000 g/mol) was obtained from Union Carbide Corporation (USA). Stannous 2-ethylhexanoate, sucrose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4′,6-diamidino-2-phenyindole dilactate (Dapi) and TritonX-100 were purchased from Sigma–Aldrich (D). Polyclonal anti-AIF was from Santa-Cruz Laboratories. sc-9416 Cys (F) and anti-L-DNase II were prepared in the laboratory as described (Torriglia et al., 1995). Anti-microtubule-associated protein light chain (MAP-LC3) was from Cell Signalling (USA), and anti-active caspase 3 was from BD Pharmingen (USA). The medium for cell culture, balanced salt solution, and phosphate buffer were obtained from Gibco (USA). Cyclosporin A was purchased from Dynapharm Distribution (CH). MilliQ water was always used in each experiment. Analytical grade acetone was purchased from Fluka (D), HPLC-grade methanol from VWR (F).

2.2. Synthesis and characterization of MPEG-hexPLA

The methoxy poly(ethylene glycol)-poly(hexyl-lactide) (MPEG-hexPLA) copolymers were synthesized by ring-opening polymerization (ROP) using stannous 2-ethylhexanoate as a catalyst by the procedure described by Trimaille et al. (2006). ^{1}H NMR was used to determine the degree of polymerization (DP) using a Bruker spectrometer (300 MHz (D)) and $CDCl_3$ as the solvent. Molecular weight (Mw) and polydispersity (PI) were analyzed by Gel Permeation Chromatography (GPC). GPC was carried out on a Waters chromatography system mounted with Styragel HR 1–4 columns (Waters, USA) and connected to a Waters 410 differential refractometer. The flow rate was set at 1 mL/min, and tetrahydrofuran was the continuous phase. Polystyrenes of known molecular weights were used as standards for calibration.

2.3. Preparation and characterization of MPEG-hexPLA micelle formulations

Unloaded polymeric micelles were prepared by the co-solvent method, as described previously (Trimaille et al., 2006; Di Tommaso et al., 2010). Briefly, 40 mg of MPEG-hexPLA copolymer (Mw ~5500 g/mol) were dissolved in 2 mL of acetone and added dropwise under sonication (10% of amplitude for the probe) (Digital Sonifier 450, Branson, USA) to 4 mL of 10 mM phosphate buffer containing 10% sucrose. The acetone was subsequently evaporated under reduced pressure at room temperature, yielding a micelle solution with a final copolymer concentration of 10 mg/mL.

The pH was measured with a pHmeter 209 (Hanna Instruments, USA), and the isotonicity was measured with a 5500 Vapor Pressure Osmometer (Wescor, USA) using Wescor calibration solutions of sodium chloride at 100, 290 and 1000 mOsm/kg (distributed by EliTechGroup; CH).

CsA loaded MPEG-hexPLA micelles were prepared with the same procedure by adding the drug to the initial organic acetone phase (0.22 $m_{CsA}/mg_{copolymer}$). After acetone evaporation, the formulations were filtered through a 0.22-μm filter under laminar flow to obtain sterile formulations. After a drug content analysis, the formulations were diluted with the aqueous phase to reach the commercial CsA concentration of 0.05% that is used in Restasis® (Allergan, USA).

The average hydrodynamic diameter and the polydispersity of the micelles were determined by Dynamic Laser Scattering (DLS) using a Zetasizer HS 3000 (Malvern Instruments, UK) with a detection angle of 90° at 25 °C after diluting the formulations with water. The mean values of three measurements of 10 runs were calculated for all samples.

The morphology of the polymeric micelles was determined by Transmission Electron Microscopy (TEM) (FEI Tecnai™ G2 Sphera, USA). Briefly, samples were deposited on copper grids, stained with 1% uranyl acetate and analyzed after drying.

The CsA content in the micelles was determined by HPLC. Samples were prepared by diluting 100 μL of micelle solution with 900 μL of mobile phase (a mixture of methanol:water (80:20) with 0.05% trifluoroacetic acid) to destroy the micelle structure and release the drug. A Waters 600 Controller, equipped with a Waters 2487 Detector (USA) and a C18 column heated at $T = 65\,^{\circ}C$, was used. The flow rate was 1.2 mL/min, and the injection volume was 20 μL. CsA was detected at 210 nm with a retention time of 6 min. A calibration curve ($R^2 = 0.9994$) was obtained with CsA standard solutions with CsA concentrations ranging from 2 μg/mL to 200 μg/mL.

2.4. Residual acetone content analysis

The residual acetone in formulations was quantified using an Agilent (USA) gas chromatography system coupled with a Hewlett Packard (USA) head-space autosampler and an Agilent methyl siloxane column. A helium flow of 1.8 mL/min was applied at a temperature of 85 °C with 1 min of equilibration time. The detector was maintained at 200 °C, and 0.8 mL of formulation were analyzed. A calibration curve using the aqueous phosphate buffer phase with acetone concentrations ranging from 50 to 10,000 ppb (ng/mL) was obtained with a $R^2 = 0.9996$. The analysis time was 5 min, and the retention time for acetone was 1.8 min.

2.5. Cell culture tests

Immortalized human corneal epithelial cells (HCE) were cultured in Dulbecco's Modified Eagle's Medium (DMEM/F-12; Gibco Life Technologies, Carlsbad, USA) supplemented with 10% decomplemented fetal bovine serum (FBS; GIBCO, USA) and 1% penicillin and streptomycin (GIBCO, USA). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were used at 80% confluency for all *in vitro* tests.

2.6. In vitro toxicity tests

The toxicity of formulations, unloaded and loaded with CsA, was tested on HCE cells using the following copolymer concentrations: 3.000, 0.300, 0.030, and 0.003 mg/mL. The latter concentration is below the critical micellar concentration (CMC), and with it the toxicity of the non-assembled MPEG-hexPLA copolymer was assessed. The corresponding CsA concentrations were 0.5000, 0.0500, 0.0050, and 0.0005 mg/mL. Ocular biocompatibility was evaluated through different tests: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability tests for studying the activation of apoptosis and autophagy by immunohistochemistry, and the clonogenic test.

For the MTT and immunohistochemistry tests, two different incubation protocols were used. Because eye drops are rapidly cleared from the surface of the eye (Lang, 1995; Jtirvinena et al., 1995), it was assumed that a 1-h incubation time would be sufficient to observe any toxic effect.

The first protocol (protocol A) consisted of 1 h of incubation followed by 3 h of recovery time before performing the tests. In the second protocol (protocol B), the cells were incubated 1 h with the formulations. The formulations were then removed, and the cells were washed with PBS (19 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM NaCl, 15 mM KCl pH 7,5) and left to recover in the incubator at 37 °C for 3 h. The cells were then incubated twice in the same sequence to simulate a conventional application of the eye drops three times a day. For the clonogenic tests, only unloaded micelles at a copolymer concentration of 3.000 mg/mL and a unimer solution with a concentration of 0.003 mg/mL (below the CMC) were used, with an incubation time of 1 h.

2.6.1. In vitro cytotoxicity test

The *in vitro* cytotoxicity of unloaded and CsA-loaded micelles at different copolymer concentrations was determined by a standard MTT test adapted from Mosmann (1983). The cells were harvested at 80% confluence, centrifuged and seeded in 24-well plates at a cell concentration of 8×10^4 cells/cm^2. The cells were left to grow in an incubator with 5% CO_2 at 37 °C for two days. On the day of the test, the medium was removed, and the cells were washed with 500 μL of PBS and then incubated with 200 μL of the micelle formulations (6 wells for each formulation). To obtain the desired copolymer concentration, a micelle formulation of 10 mg/mL copolymer was diluted using one part 10 mM phosphate buffer with 10% sucrose and two parts medium. At each incubation time point, 250 μL of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) filtered solution with a concentration of 1 mg/mL in medium were added to each well. The plates were incubated at 37 °C for 3 h to allow the soluble yellow MTT to be reduced into dark-blue, insoluble formazan crystals by the metabolically active cells. The formazan crystals were subsequently dissolved by the addition of 250 μL isopropanol at room temperature with shaking for 20 min. The UV absorbance of individual wells was measured at 570 nm with a microplate reader (Model 550, Bio-RAD, Hercules, USA). An unspecific signal of a reference wavelength (690 nm) was subtracted. A solution composed of one part 10 mM phosphate buffer with 10% sucrose and two parts medium was used as positive control (100% cell survival).

2.6.2. Immunohistochemistry test

Three hundred microliters of a cell suspension at 8×10^4 cells/cm^2 were seeded in a LabTec Chamber Slide System (Nalge Nunc International, USA). The cells were left to grow for two days before the medium was removed, the cells were washed with PBS and 200 μL of each tested formulation was added in every well and incubated for the established time points at 37 °C in a humidified atmosphere with 5% CO_2. Then, the formulations were removed, and the cells were washed two times with PBS-Ca-Mg-solution (19 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM NaCl, 15 mM KCl, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ pH 7,5) and fixed in 4% paraformaldehyde (PAF) at room temperature for 15 min. The cells were permeabilized with 0.1% Triton X100 in PBS for 30 min. After washing the cells with PBS, 1% skim milk in PBS was used for saturating non-specific fixation sites at room temperature for 1 h. The cells were incubated for 1 h with the following antibodies (1/100 dilution in 0.1% skim milk-PBS): polyclonal anti-L-DNase II, polyclonal anti-AIF, polyclonal anti-caspase 3, and polyclonal anti-LC3. The cells were washed with PBS three times and incubated with Alexa Fluor anti-rabbit IgG for the first three antibodies and with Alexa Fluor anti-goat IgG for the final antibody. The secondary antibodies were diluted 1/100 in PBS plus 0.1% skim milk, and the staining reaction lasted 1 h. The cells were washed three times with PBS, and the nuclei were stained with DAPI (1/1000 dilution) for 5 min. The cells were then washed two times and mounted in PBS/glycerol (50/50) on cover slides. The cells were examined by fluorescence microscopy using an Olympus microscope coupled with a digital camera. A magnification of 40X was used for all observations.

2.6.3. Clonogenic test

The clonogenic test was adapted from the method described by Puck and Markus (Puck and Markus, 1956). The cells were seeded in a 24-well plate at a concentration of 8×10^4 cell/cm^2, and after 2 days of growth at 37 °C with 5% of CO_2, the cells were washed and exposed to 200 μL of the formulation. Six wells were used for every formulation. After 1 h of incubation, the cells were washed with PBS and left to recover in the incubator at 37 °C for 3 h. The cells were then harvested, and the cells treated with the same for-

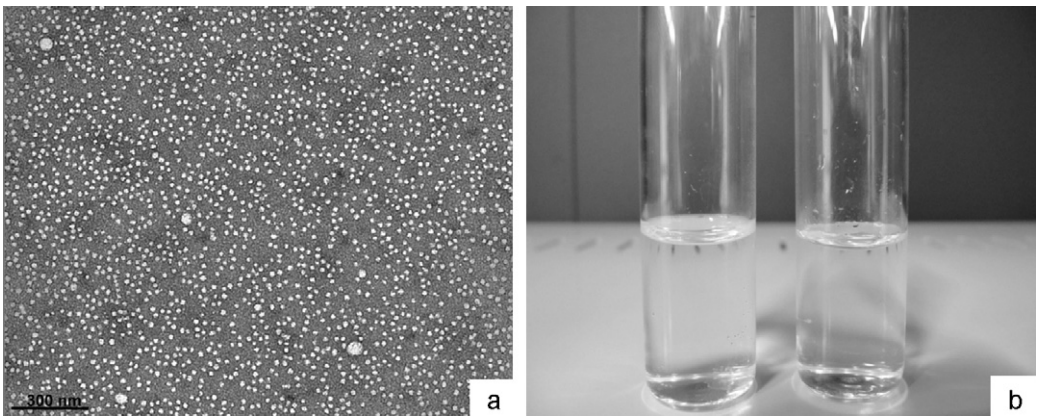

Fig. 1. (a) TEM picture of CsA/MPEG-hexPLA loaded micelles and (b) 0.5% CsA/MPEG-hexPLA micelle formulation on the left compared with water on the right side.

mulation were collected and seeded in four 96-well plates at very low cell concentrations (approximately 1 cell per well). The cells were stored at 37 °C in the incubator with 5% CO_2 and observed daily under an inverted microscope to assess colony formation.

2.7. In vivo ocular tolerance

The ocular tolerance test was carried out on female albino New Zealand rabbits (4–5 kg weight) without ocular damage. The experimental protocol was approved by the Canton of Geneva's Local Ethics Committee for Animal Experimentation (1020/3525/1).

The ocular tolerance of unloaded and CsA loaded micelle formulations for a copolymer concentration of 3 mg/mL (with a CsA concentration of 0.05% for drug loaded micelles) was evaluated on rabbit eyes by CLSO, as reported in previous studies (Felt et al., 1999; Baydoun et al., 2004; Lallemand et al., 2005). Briefly, 50 μL of the formulation was applied topically in the cul-de-sac of the right eye four times a day for three days, with a 2.5-h interval between the instillations. Only one instillation was performed on the fourth day before the ophthalmoscopic observation. Ten minutes after the last formulation instillation, 25 μL of a sterile and isotonic 0.5% fluorescein solution was applied on the cornea to allow the fixation of dead cells on the corneal surface, and the eye was washed with a 0.9% saline solution 2 min later. The eyes were analyzed with a CLSO (Zeiss, D) modified as previously described (Furrer et al., 2002). The obtained fluorescent images were treated with an image processing system (Semper6, Synoptics, UK). The fluorescent ocular surface injured by the formulations, which could provoke the death of corneal epithelial cells if toxic, was expressed as a percentage of the total corneal surface and reported on a tolerance evaluation scale as previously established (Kaelin, 1994). Each formulation was tested on four rabbits.

2.8. Statistics

For MTT tests and CLSO tests, comparisons were made with Student's *t*-test (unpaired samples). Samples were determined to be significantly different from each other when $P < 0.05$.

3. Results

3.1. Preparation and characterization of MPEG-hexPLA micelle formulations

The MPEG-hexPLA micelle formulations were prepared with a MPEG-hexPLA copolymer with a molecular weight of 5570 g/mol and a polydispersity index (PI) of 1.12.

The micelle formulations had a pH of 7.15, were isotonic with a value of 292 ± 2 mOsm/kg, and were perfectly transparent comparable to water (Fig. 1).

The micelle average hydrodynamic diameter (Z_{av}) was 37.4 ± 0.1 nm with a PI of 0.4 for unloaded micelles, and 51.4 ± 0.4 nm with a PI of 0.3 for CsA-loaded micelles.

TEM analysis showed that the micelles were spherical and homogenous, and no aggregates were present. No differences were observed between loaded and unloaded micelles in terms of morphology. The particle size visualized by TEM was the same as the size obtained by DLS.

The CsA incorporation ratio was approximately 20% (w/w), with a concentration of 1.5 mg/mL in the final formulation.

3.2. Residual acetone content analysis

The residual acetone amount in the final formulations was measured by gas chromatography and was found to be 19 ± 6 μg/mL (ppm) and 30 ± 4 μg/mL (ppm) for unloaded and CsA-loaded micelle formulations, respectively.

3.3. In vitro toxicity tests

All *in vitro* toxicity tests of the MPEG-hexPLA micelle formulations were evaluated on a HCE cell line. The results are presented in detail in the following sections.

3.3.1. Cytotoxicity test

The results of cell survival after treatment with unloaded and CsA-loaded micelle formulations are presented in Figs. 2 and 3. After 1 h of incubation (Fig. 2), no cell death was detected for any concentration of copolymer or for either formulation. Cell survival was 100%, and no significant difference was noted compared to the positive control.

For the second MTT test with an incubation time of 1 h repeated 3 times in a day, different results were obtained (Fig. 3).

For the formulations prepared with the high copolymer concentration of 3.000 mg/mL, no toxicity was observed, and cell survival was not significantly different from the control. When the copolymer concentration was decreased, a decrease in cell survival was observed. The lowest copolymer concentration of 0.003 mg/mL (below the CMC) seemed to be more toxic because it reduced the percentage of cell survival to approximately 60% of the whole cell population.

Concerning the formulations containing CsA, a decrease in cell survival was obtained with copolymer concentrations of 0.300 and 0.030 mg/mL, with approximately 70% and 60% of cells surviving, respectively. These results were significantly different from the

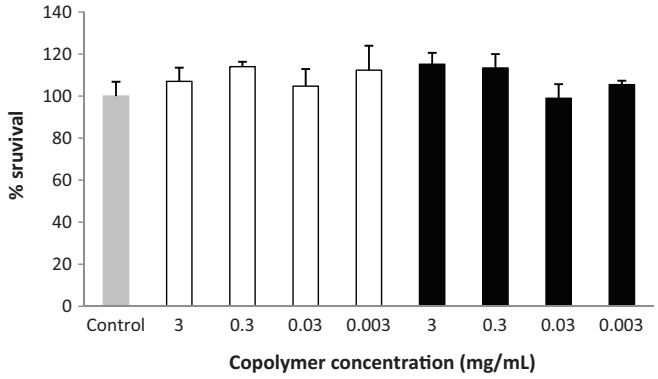

Fig. 2. MTT results after 1 h of incubation with formulations. Grey column is the control (constituted of one part 10 mM phosphate buffer with 10% sucrose and two parts of cell growth medium), white columns are the unloaded micelles and black columns are CsA loaded micelles. $n = 6$, values ± SD.

positive control. However, treatment with the lowest copolymer concentration of 0.003 mg/mL was not significantly different from the control.

3.3.2. Immunohistochemistry; activation of cell death

After incubation with MPEG-hexPLA formulations, the activation of enzymes responsible for apoptosis was studied by immunohistochemistry analysis. Considering that apoptosis could be caspase-dependent (Taylor et al., 2008) or caspase-independent (Abraham and Shaham, 2004; Torriglia et al., 2008), the activation of caspases was assessed by evaluating the activation of anti-caspase-3. Two caspase-independent mechanisms were evaluated: the LEI/L-DNase II and the AIF pathways. Furthermore, because autophagy is a protective mechanism that could lead to cell death (Gozuacik and Kimchi, 2007), the activation of this pathway was evaluated through the localization of the LC3 protein.

The results concerning the anti-AIF and the anti-IL-DNase II staining after 1 h of incubation with the formulations are presented in Fig. 4.

As shown in Fig. 4, there was no activation of these enzymes, and their location was not nuclear. These results indicate that the unloaded and CsA-loaded micelles at all tested concentrations were not activating the cellular mechanisms that lead to caspase-independent apoptosis under the applied conditions. Using both protocols, both immunohystochemistry tests (Fig. 5) showed the same results.

Concerning the caspase-3 study, no activation was detected after both incubation protocols were used with unloaded and CsA-loaded MPEG-hexPLA micelles or with MPEG-hexPLA unimer solutions.

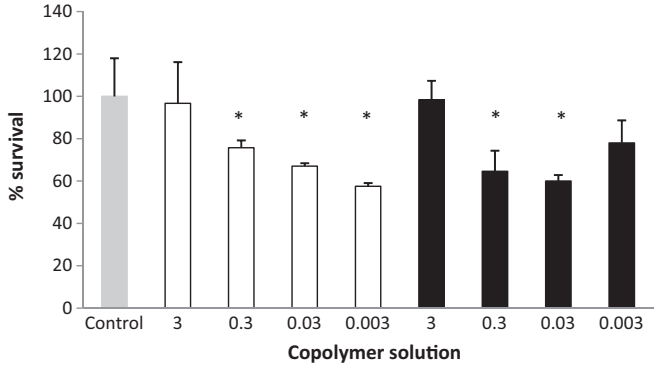

Fig. 3. MTT results after 1 h of incubation repeated 3 times in a day with formulations. Grey column is the control (constituted of one part 10 mM phosphate buffer with 10% sucrose and two parts of cell growth medium), white columns are the unloaded micelles and black columns are CsA loaded micelles. $n = 6$, values ± SD.

The microtubule-associated protein 1 light chain 3 (LC3) is an autophagosome marker that is activated during autophagy (Tanida et al., 2008). Autophagy implies the presence of intracellular vesicles with a double membrane (Reggiori and Klionsky, 2002). After a 1-h incubation time, some autophagy vesicles were present when cells were treated with a pure unimer solution (Fig. 6).

However, no punctuated labeling was observed after incubation with a unimer solution when using protocol B (incubation repeated three times in one day).

3.3.3. Clonogenic tests

Clonogenic tests were carried out to confirm the results obtained from the other *in vitro* tests. After treatment with the formulations, the cells were seeded at a very low concentration (approximately 1 cell/well) to test their replication capacity. Four 96-well plates were used for each tested formulation. The colony was visible after four days in every well, with 100% of cells proliferating after treatment with unloaded micelles or with a unimer solution.

3.4. Ocular tolerance

The ocular tolerance was evaluated on rabbit eyes by CLSO. The ocular surface damaged by the formulations was stained with a fluorescein solution. The percentage of the fluorescent injured ocular surface was 5.19 ± 2.20% and 5.63 ± 2.82% for unloaded and CsA-loaded micelles, respectively (Fig. 7).

Compared to a 0.9% NaCl solution, for which the percentage of fluorescent injured ocular surface was 1.15 ± 0.36% (Furrer et al., 2000), both micelle formulations were not significantly different from each other and from the control.

4. Discussion

Because of the rapid development of nanotechnologies for pharmaceutical and medical applications, it is necessary to adequately evaluate the potential toxicity of novel materials with nanosize features before any clinical use can be considered. The physicochemical characteristics of nanosized drug carriers are important for their possible interactions with cells and tissues and, consequently, for possible toxicity issues. Nano-sized materials can be taken up and internalized by cells, and an accumulation of these materials could cause adverse effects. In addition, biodegradable materials may accumulate in cells and cause intracellular changes (Lewinski et al., 2008). The current study presented novel degradable MPEG-hexPLA polymeric micelles that are drug carriers with a size of about 50 nm. These micelles could facilitate better corneal penetration compared to standard eye drops and may reduce the required number of eye-drop applications. Moreover, the investigated formulations are very clear and transparent solutions, which may help avoid the problems of blurred vision and discomfort for the patient after topical applications. Finally, these micelles showed a high capacity to efficiently incorporate hydrophobic drugs such as CsA. However, before evaluating their *in vivo* ability to enhance corneal penetration of this novel drug carrier system, its ocular biocompatibility must be evaluated. Any tissue disruption due to possible toxicity of the formulations would give incorrect results regarding the corneal penetration performance. Therefore, the MPEG-hexPLA micelles were tested here for their ocular biocompatibility.

First, all prepared formulations were analyzed for their residual acetone content. Certainly, the residual solvent in a pharmaceutical formulation must be at an acceptable level for the safety of the patient. Acetone, a class 3 solvent in the European Medicin Agency (EMA) guidelines for residual solvents, is considered to have

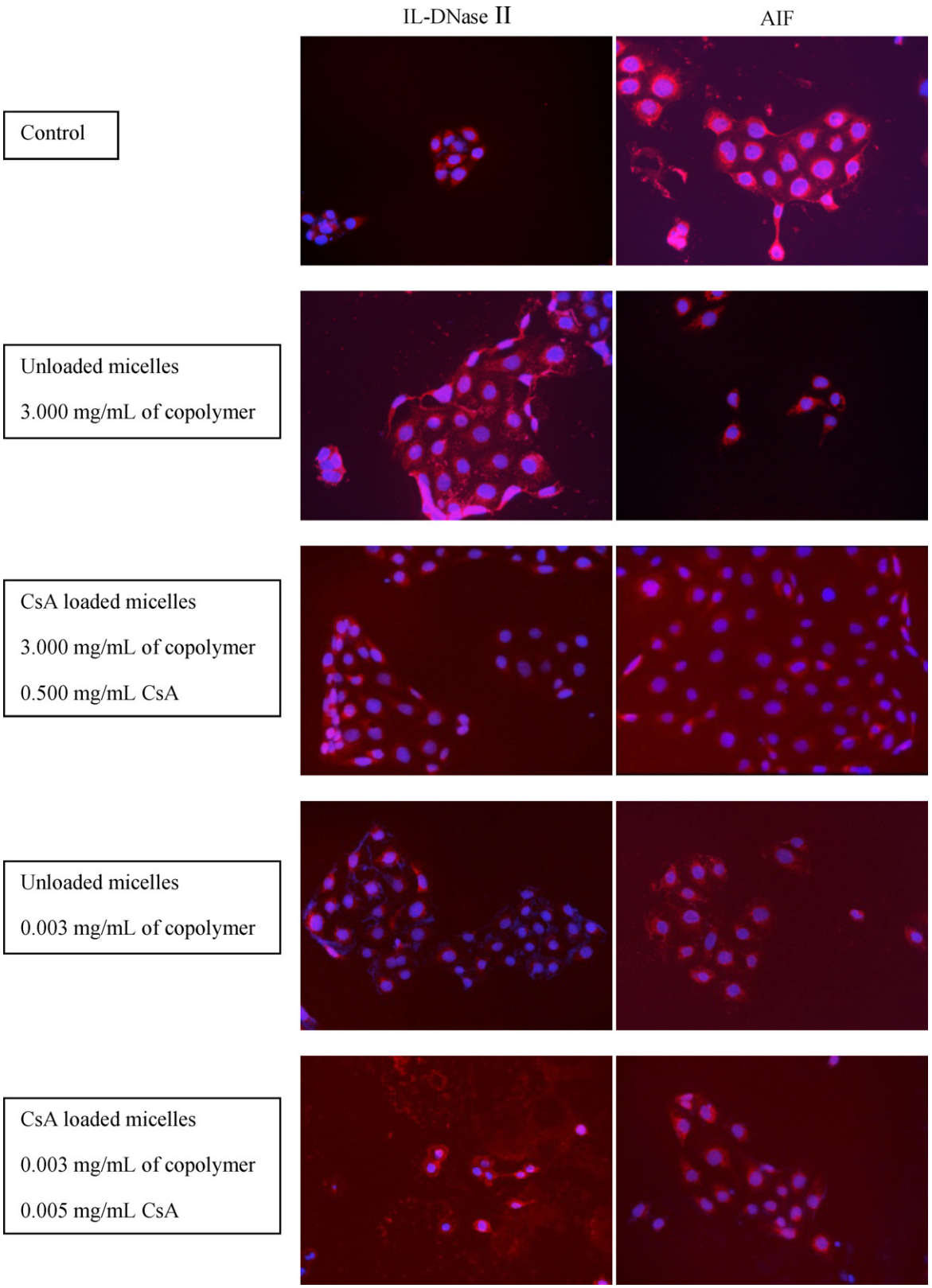

Fig. 4. Indirect immunofluorescence of IL-DNase II and AIF after 1 h of incubation. Blue: nuclei stained with DAPI. Red: antibody under investigation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

a low toxic potential to humans. The permitted daily exposure for a class 3 solvent is 50 mg per day (corresponding to 5000 ppm) (Impurities: guidelines for residual solvents, Q3C(R4), EMA, February 2009). Considering that the novel formulation is intended for topical ophthalmic use, it is important that the residual acetone amount is as low as possible to avoid injury to the eye. At high vapor concentrations, 500 ppm acetone were found to cause some burning sensation in the eye (Nelson et al., 1943). One drop of ace-

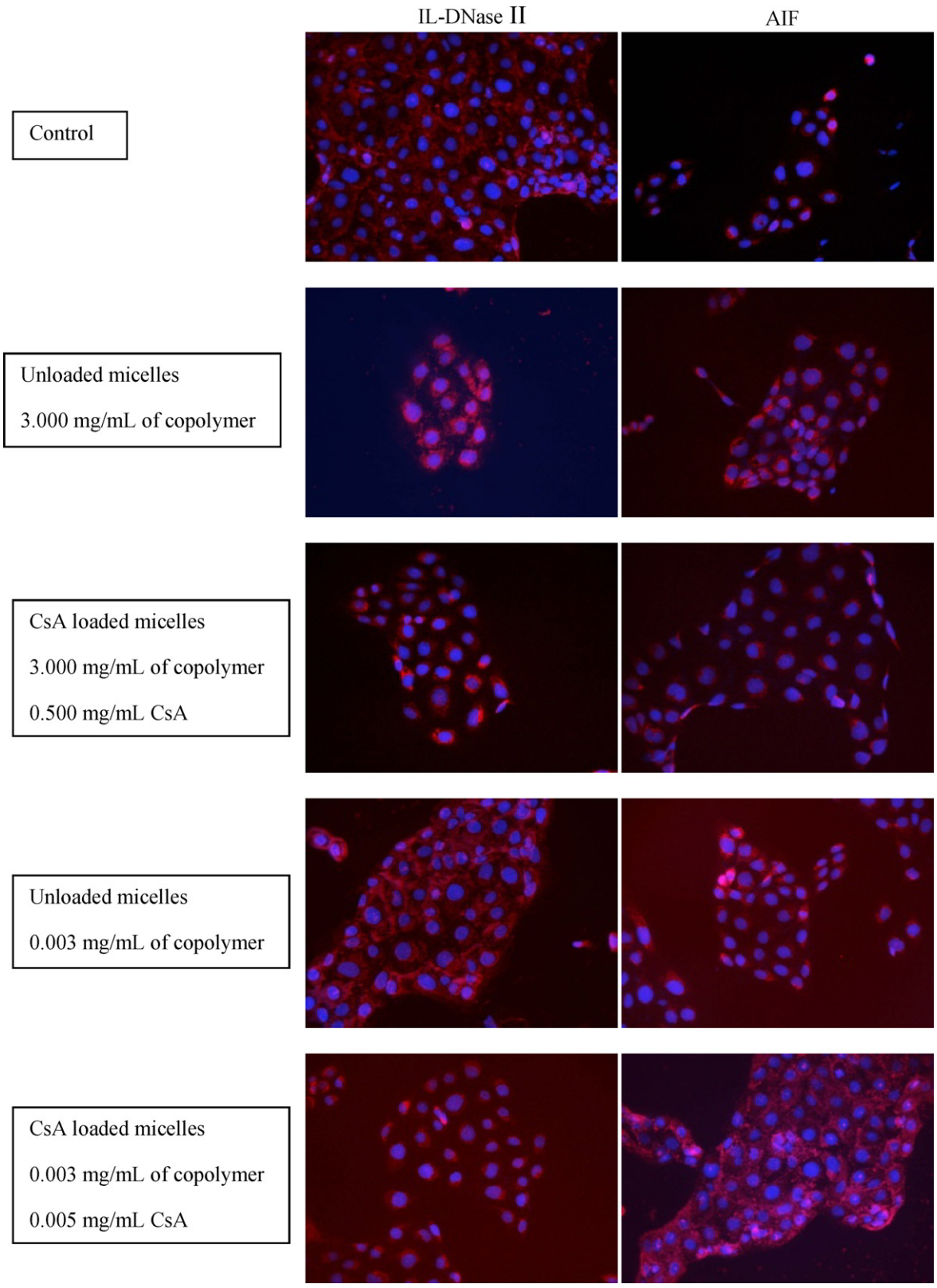

Fig. 5. Indirect immunofluorescence of IL-DNase II and AIF after 1 h of incubation repeated three times. Blue: nuclei stained with DAPI. Red: antibody under investigation LC3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

tone applied on the human eye caused a stinging sensation, but if promptly washed, the injury was limited only to the corneal epithelium with a complete recovery after one or two days (McLaughlin, 1946). Additionally, in the case of rabbit eyes, the injury was reversible when a drop was topically applied (Carpenter and Smith, 1946). There is no clear evidence of injury to the optic nerve, of the induction of a cataract, or of lens abnormalities resulting from acetone treatment (Grant, 1974). Here, the prepared CsA/MPEG-

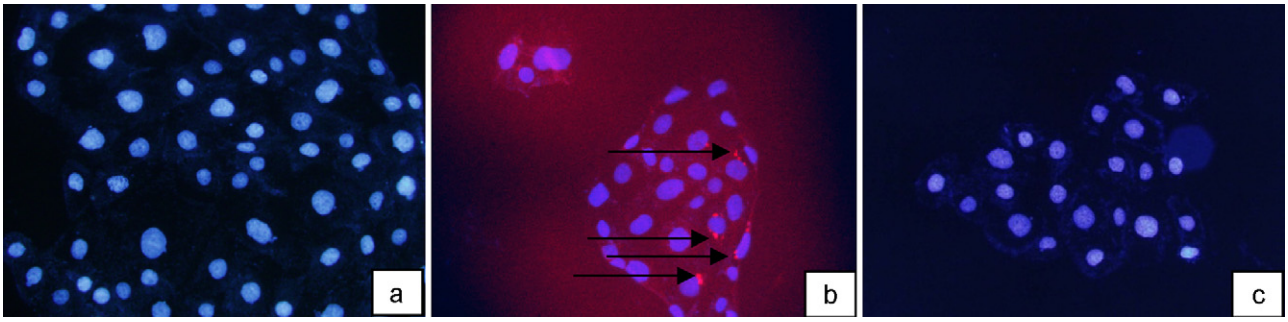

Fig. 6. Indirect immunofluorescence for LC3 antibody. (a) Control, (b) unloaded 0.003 mg/mL, (c) CsA loaded 0.003 mg/mL. Blue: nuclei stained with DAPI. Red: antibody under investigation. Arrows indicate the autophagy vesicles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

hexPLA formulations had a very low residual acetone content of 30 ± 4 μg/mL, which corresponds to 1.5 μg of acetone in one 50-μL drop of the formulation. This quantity is not expected to provoke damage to the eye. In conclusion, the applied formulation procedure is appropriate for preparing these novel micellar eye drops.

Moreover, no micelle aggregation was noted in these formulations when stored at 4 °C for over one year (Di Tommaso et al., 2010). This property is of high importance because aggregation is often considered to be one of the variables of nanoparticle toxicity (Jones and Grainger, 2009).

The *in vitro* toxicity towards an eye-drop application was assessed on HCE cells using different tests. The surface cells of the corneal epithelium are relatively impermeable and are the first barrier to foreign substances, but these cells are vulnerable to trauma caused by foreign substances.

The MTT test is widely used for assessing the cytotoxicity and biocompatibility of materials (Wan et al., 1994). The MPEG-hexPLA micelle formulations and unimer solutions did not affect cell viability after 1 h of incubation. After three 1-h incubations during one day, with 3 h of recovery between applications, the formulations containing a therapeutic dose of CsA with a copolymer concentration of 3.000 mg/mL did not affect the cell viability. A 100% cell survival was also measured for the positive control. A decrease in cell viability was observed when the cells were treated with a unimer solution or with formulations containing a copolymer concentration close to the CMC. This could be explained by an interaction between the hexPLA polymer chains (when not assembled in micelles) and the cell membrane, concomitant with a destabilization of the membrane. However, this could also be due to a removal of cells during the test because the three different treatments on the same day correspond to three different cell manipulations. Furthermore, good results were obtained with the immunohistochemistry tests for cells treated with protocol B. Due to the very low CMC of the MPEG-hexPLA copolymer (8 mg/L) (Trimaille et al., 2006) and the stability of the micelles, unimers should not be present at the surface of the eye after topical application of formulations with appropriate concentrations.

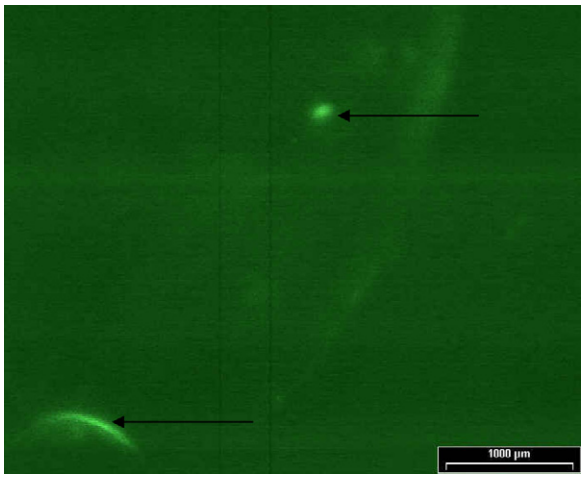

Fig. 7. Corneal surface of a rabbit treated with CsA/MPEGhexPLA micelles with an injured corneal surface of 2.43%. Arrows indicate the dead corneal epithelial cells stained by fluorescein.

Immunohistochemistry is widely used to study the cellular mechanisms of enzymes to better understand the functioning of cells (Tallury et al., 2010). Here, this potent technique was used to verify the results obtained by the MTT tests. The immunohistochemistry demonstrated the activation of several effectors of apoptosis and autophagy. Leukocyte elastase inhibitor (LEI) is a serine protease inhibitor located in the cytoplasm, which under specific conditions (e.g., acidic pH or oxidative stress) is activated into L-DNaseII, a nuclear protein responsible for DNA degradation (Torriglia et al., 1998; Padron-Barthe et al., 2007). Apoptosis inducing factor (AIF) is a mitochondrial oxidoreductase. During apoptosis of programmed necrosis, AIF is released and translocated into the nucleus, where it leads to chromatin condensation and DNA condensation (Boujrad et al., 2007). The caspases are a family of proteases that play an important role in apoptosis. Caspase 3 is an effector caspase which is cleaved and activated during apoptosis (Chang and Yang, 2000). Here, the immunohistochemistry studies showed that L-DNase II, AIF and caspase 3 were not activated, indicating that no caspase-dependent or caspase-independent apoptosis was induced after treatment with micelles or unimer solutions. These results support the compatibility of these formulations. Another effect of cell reactivity is the induction of autophagy, a pathway related to either cell survival or cell death depending on the cellular conditions. This pathway was investigated through the cellular location of LC3, a protein that translocates to the autophagosome wall after the induction of autophagy. When both protocols were applied, no detection of this lipid binder was observed after treatment with either formulation at a copolymer concentration of 3 mg/mL. This copolymer concentration is present in 0.05% CsA formulations, which is the same concentration used in Restasis®. These results showed that the MPEG-hexPLA micelle formulations did not induce autophagy in HCE cells. The presence of autophagy vesicles when the cells were treated with the unimer solution was observed by staining for the LC3 enzyme. However, this activation is not a clear sign of toxicity because autophagy is considered to be both a cell survival and a cell death mechanism (Gozuacik and Kimchi, 2007). Furthermore, the activation of this enzyme was detected only during the tests that used 1 h of cell incubation with a unimer solution. In contrast, three applications per day did not result in any LC3 lipidation, indicating that autophagy activation was transient and that the cells were

able to remain alive. A control experiment of applying unimers with added CsA showed no activation of autophagy, indicating that the CsA could play a protective role for the cells.

In addition, a clonogenic test that was first described by Puck and coworkers (Puck and Markus, 1956) was carried out. This test is widely used in the evaluation of the activity of anticancer agents or to determine the effect of radiation on cells (Brown and Attardi, 2005). Recently, it has been proposed as a reliable test for the evaluation of the toxicity of carbon-based nanomaterials. Because it is not based on the use of a colorimetric dye, as is usually used in cytotoxicity tests, the falsifying interactions of dyes with nanosized materials are avoided (Herzog et al., 2007) due to the fact that the exposed cells are directly observed and counted. The results obtained for the MPEG-hexPLA formulation showed the capacity of the cells to continue the mitotic cycle, which proves the absence of toxicity and which would have led to a stoppage of cell proliferation in the other case.

In summary, all of the *in vitro* tests revealed that the MPEG-hexPLA micelle formulations are biocompatible and should be suitable for topical ocular application. Considering the stability of the micelles and the formulation concentrations, side effects by unimers should not be present during instillation on the corneal surface.

Finally, an *in vivo* evaluation of ocular tolerance was conducted on rabbit eyes using the CLSO technique. Here, the unloaded and CsA-loaded micelle formulations were instilled four times per day in a manner similar to everyday eye-drop application. With a damaged corneal surface of less than 20%, the MPEG-hexPLA micelle formulations were considered to be well tolerated and similar to the control. The measured surface damage by the control after treatment with a 0.9% NaCl solution is due to the physiological desquamation process of the corneal surface (Furrer et al., 2000). These results indicate that the new formulations should be pharmaceutically acceptable.

5. Summary and conclusions

The *in vitro* and *in vivo* ocular biocompatibility of the novel MPEG-hexPLA formulations was demonstrated, and their non-toxicity on HCE cells was proven by MTT and clonogenic tests. Moreover, immunohistochemistry analysis demonstrated that apoptosis mechanisms were not activated in the cells. Furthermore, the CLSO studies showed an excellent topical ocular tolerance of the micelles that was not different from treatment with a 0.9% NaCl solution.

The ocular biocompatibility, the transparency and the stability of MPEG-hexPLA micelles indicate that the tested CsA formulation may be used as possible eye drop formulations. The drug dose can be easily adjusted for the CsA treatment of specific diseases, such as dry eye syndrome, autoimmune uveitis, or the prevention of corneal graft rejection. Due to the physico-chemical characteristics of the presented micelle carriers, the CsA bioavailability may be increased by a simple topical instillation. Moreover, in addition to these investigated CsA formulations, other potent hydrophobic drugs for the treatment of ophthalmic diseases could be considered.

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