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Sustained-release hydrogels of topotecan for retinoblastoma

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ABSTRACT

Treatment of retinoblastoma, the most common primary ocular malignancy in children, has greatly improved over the last decade. Still, new devices for chemotherapy are needed to achieve better tumor control. The aim of this project was to develop an ocular drug delivery system for topotecan (TPT) loaded in biocompatible hydrogels of poly(ε-caprolactone)-poly(ethyleneglycol)-poly(ε-caprolactone) block copolymers (PCL-PEG-PCL) for sustained TPT release in the vitreous humor.

Hydrogels were prepared from TPT and synthesized PCL-PEG-PCL copolymers. Rheological properties and *in vitro* and *in vivo* TPT release were studied. Hydrogel cytotoxicity was evaluated in retinoblastoma cells as a surrogate for efficacy and TPT vitreous pharmacokinetics and systemic as well as ocular toxicity were evaluated in rabbits. The pseudoplastic behavior of the hydrogels makes them suitable for intraocular administration. *In vitro* release profiles showed a sustained release of TPT from PCL-PEG-PCL up to 7 days and drug loading did not affect the release pattern. Blank hydrogels did not affect retinoblastoma cell viability but 0.4% (w/w) TPT-loaded hydrogel was highly cytotoxic for at least 7 days. After intravitreal injection, TPT vitreous concentrations were sustained above the pharmacologically active concentration. One month after injection, animals with blank or TPT-loaded hydrogels showed no systemic toxicity or retinal impairment on fundus examination, electroretinographic, and histopathological assessments. These novel TPT-hydrogels can deliver sustained concentrations of active drug into the vitreous with excellent biocompatibility *in vivo* and pronounced cytotoxic activity in retinoblastoma cells and may become an additional strategy for intraocular retinoblastoma treatment.

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1. Introduction

Retinoblastoma is the most common intraocular tumor in children [1]. Although significant improvements have been achieved over the past few years in terms of ocular survival, tumor recurrence in the vitreous is associated with worse ocular survival and remains a challenge [1]. The need to improve outcome is essential, especially for children with bilateral disease in whom both eyes are compromised with tumor and enucleation would lead to complete blindness [2].

An ideal scenario would be the use of an active chemotherapeutic agent delivered through a local route devoid of retinal and systemic toxicity that controls tumor growth while avoiding relapses. Topotecan (TPT) is a topoisomerase-I inhibitor that causes single-strand breaks in DNA and thereby interferes in cell replication, with extensive and well-documented *in vitro* and *in vivo* activity against retinoblastoma [3,4]. The antitumor efficacy is highly dependent on the drug and the schedule of drug administration [5]. The protracted administration of TPT in a five days on followed by two days off schedule has shown better tumor control and fewer severe systemic adverse effects than the single-dose administration of high-dose TPT in xenograft animals bearing pedi-

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atric tumors and in children with solid tumors [6–8]. Specifically for ocular purposes, a favorable and prolonged vitreous TPT disposition of has been demonstrated after intravitreal injection of a single-dose aqueous solution of TPT in rabbits [7]. Although high vitreous exposure was attained using this local route, no retinal toxicity was found on electroretinographic and histopathological assessment in the animal model [7]. In this setting, TPT emerges as an ideal candidate for retinoblastoma treatment if incorporated in a sustained-drug-release device that allows for protracted delivery.

Different approaches have been studied for chemotherapy delivery into the vitreous humor of eyes with tumors. Periocular administration of an aqueous solution [8,9] or an episcleral implant [10] resulted in low TPT bioavailability in the vitreous of the treated eyes of rabbits and little benefit in terms of ocular survival in children [8]. Further research in the periocular administration of TPT using controlled-release devices, such as fibrin sealant, showed limited use in less advanced intraocular tumors probably due to low bioavailability in the vitreous due to rapid orbital clearance and limited *trans*-scleral penetration of TPT [11]. The development of a safe technique for intravitreal injection of chemotherapy preventing extraocular seeding of tumor cells marked a new era of retinoblastoma treatment and permitted to save eyes that were enucleated upfront in the past [12]. Nonetheless, its main shortcoming has been the need for weekly intravitreal injections of chemotherapy for tumor control. To overcome this limitation, a sustained-release formulation of TPT for intravitreal injection had to be developed.

Different materials, such as liposomes [13], lipid nanoparticles [14], polymeric implants [10], and hydrogels [15], have been studied for sustained TPT release, but none of them was indicated for ophthalmic applications. Disadvantages of previously developed liposome formulations of TPT included a low loading efficiency and a rapid elimination in animal models [13]. Others developed lipid nanoparticles of TPT with a promising cytotoxic effect but no available data on the toxic effect of the nanoparticle itself was published [13]. We hypothesized that PCL polymer, a commercial and FDA-approved inactive ingredient already employed in drug delivery systems, could be suitable for a sustained-release formulation of TPT based on its well-documented biocompatibility [16] and versatility to entrap hydrophilic drugs [15]. Moreover, the low cost of PCL favors its use allowing more affordable novel developments to be translated into the clinics of retinoblastoma.

Therefore, we aimed to design a TPT-loaded hydrogel to deliver sustained and pharmacologically active levels to the vitreous humor using a single intravitreal injection. We developed different biocompatible TPT-loaded hydrogels composed of PCL-PEG-PCL copolymer and characterized the physicochemical properties and the *in vitro* release of TPT from the hydrogels. We also studied the vitreous TPT disposition after intravitreal injection in rabbits and the potential toxicity of the drug and the blank hydrogel in the retinal tissue of the animals. In addition, we assessed the antitumor activity in retinoblastoma cell lines as a parameter of efficacy. Thus, the present is a multimodality approach to the development and *in vivo* and *in vitro* characterization of a new sustained-delivery TPT formulation with potential translation into the clinical treatment retinoblastoma.

2. Materials & methods

2.1. Materials

Poly(ethylene glycols) of different molecular weights (1 kg/mol, PEG1000; 4 kg/mol, PEG4000; 6 kg/mol, PEG6000) were provided by Merck Chemicals (Buenos Aires, Argentina), ε-caprolactone 99% (monomer, CL, Sigma, Argentina), tin (II) 2-ethyl-hexanoate 95% (catalyst, SnOct, Sigma, Argentina). TPT hydrochloride was kindly donated by Asofarma S.A. (Buenos Aires, Argentina). Stock solutions of TPT were prepared in methanol and stored at −20 °C to minimize degradation. High-performance liquid chromatography (HPLC) solvents (Sintorgan, Buenos Aires, Argentina) were used. HPLC-grade water was obtained using a Milli-Q system (Millipore Corporation (Billerica, MA). Multi-use floating dialysis bags (DispoDialyzer®) were purchased from Spectrum Labs (USA).

2.2. Synthesis of PCL-PEG-PCL copolymer

The three PCL-PEG-PCL derivatives were synthesized by ring opening polymerization (ROP) of CL in the presence of PEG (PEG1000, PEG4000 or PEG6000) catalyzed by SnOct and assisted by microwave radiation as described elsewhere [17]. Briefly, PEG was poured in a round-bottom flask (250 mL) and dried under vacuum conditions at 80–90 °C over 2 h in a glycerin bath. Then, the adequate amount of CL (10% molar excess) and the catalyst were added and mixed. Subsequently, the reaction mixture was poured inside a household microwave apparatus (Whirlpool®, WMD20SB, microwave frequency 2450 MHz, potency 800 W, Argentina) connected to a condenser where it was exposed to microwave radiation for 10 min under reflux. Finally, the crude was dissolved in dichloromethane (50 mL) and precipitated in 200 mL of *n*-hexane. The derivatives were isolated by filtration, washed with petroleum ether (40–60 °C) and dried at room temperature until constant weight. Copolymers with different average molecular weight were obtained varying the PEG molecular weight and the total amount of CL employed.

2.3. Copolymer characterization

Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained from deuterated chloroform (Sigma) solutions at room temperature on a Bruker MSL300 spectrometer (Karlsruhe, Germany) at 300 MHz. The hydrophobic/hydrophilic balance, as represented by the CL/ethylene oxide (EO) molar ratio, and the number-average molecular weight (Mn) of the different copolymers were calculated by rationing the integration area of the peaks of PCL protons (2H, triplet, 2.30 ppm) and PEG (4H, multiplet, 3.65 ppm). Number- and weight-average molecular weights (Mn and Mw) and molecular weight distributions (Mw/Mn polydispersity, PDI) were determined by gel permeation chromatography (GPC) using a Waters GPC instrument (Berlin, Germany) provided with a refractive index detector (Waters 2414), a Waters Styragel HR4 THF 7.8 × 300 mm column, and tetrahydrofuran as eluent. Polystyrene standards (Polymer Laboratories, Shropshire, UK) were used for calibration.

2.4. Preparation of TPT-blank and TPT-loaded hydrogels

In a first step, each PCL-PEG-PCL copolymer synthesized (35% w/w) was suspended in a deionized sterile water solution in a glass beaker with constant magnetic stirring (50 RPM) for 4 h at room temperature. Then, the suspension was heated at 65 °C for 1 min with gentle magnetic stirring. Once the copolymer gelation was completed, the stirring was continued until the gel cooled to room temperature (15 min) as previously described. In a second step, to evaluate the *in vitro* behavior of TPT-loaded hydrogels, they were prepared with three different loads (0.05, 0.1, and 0.2 mg TPT/g of hydrogel). Aqueous solutions of TPT were prepared by direct dissolution of commercial TPT (Topokebir®, Aspen, Argentina) [16,18].

2.5. Rheological analyses

Rheological behavior of the hydrogels prepared with the synthesized copolymers was analyzed using a rotational type viscometer

(Brookfield Rotational Viscometer RVT with Spindle #2, Massachusetts, USA) equipped with a temperature controller. The viscosity of samples was measured in units of milliPascal × seconds (mPa s) at 25 °C. Previously, the sample was placed in the sample container and kept for 5 min so that it reached a constant temperature.

2.6. *In vitro* TPT release study

In vitro TPT release assays from the hydrogels prepared with each derivative (employing three different drug-load levels) were performed using the dialysis method over 168 h. Samples (2 g of hydrogel) were placed into a dialysis bag (regenerated cellulose dialysis membranes; molecular weight cut-off of 3500 g/mol; Spectra/Por® 3 nominal flat width of 18 mm, diameter of 11.5 mm, and volume/length ratio of 1.1 mL/cm; Spectrum Laboratories, Inc., Rancho Dominguez, California, USA), sealed, and placed in a Falcon® conical tube (50 mL) containing the release medium (PBS, pH 7.4, 40 mL). Then, each Falcon® conical tube was incubated at 37 °C. At different time points (0.5, 1, 3, 5, 8, 24, 48, 72, 96, 120, 144, and 168 h), the complete volume was withdrawn and replaced with an equal volume of fresh medium pre-heated at 37 °C. Released total TPT was assayed using HPLC coupled with fluorometric detection previously developed and validated in our laboratory [7].

2.7. *In vitro* cytotoxicity study

Cytotoxicity studies were performed in WERI-RB1 cells, obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 culture media (Invitrogen) with various supplements as previously described [19].

To assess the cytotoxic activity of the hydrogel without load (blank hydrogel) or with TPT, retinoblastoma cells were seeded in 24-well plates at a density of 10^5 cells per well and allowed to grow for 24 h. Afterwards, 30 μL of blank or TPT-loaded hydrogel was placed in the upper chamber of polycarbonate transwell inserts (8-μm pore size, Corning, NY). Each treatment condition was assayed in triplicate. Following 1, 3, and 5 h of incubation, the transwells were removed and cell proliferation was determined after 72 h. In order to assess for prolonged TPT release and activity, the removed transwells were introduced in 24-well plates containing 1 mL of fresh culture medium without cells. After 24, 48, 72, 96, and 168 h of contact, the transwells were transferred to fresh 24-well plates seeded with WERI-RB cells and allowed contact for 24 h. Thereafter, the transwells were discarded and cell proliferation was determined after 72 h.

In all cases, cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA) as detailed elsewhere [20].

2.8. *In vivo* pharmacokinetic and safety studies

New Zealand albino rabbits (weighing 1.5–2.9 kg) were randomly assigned to two groups for pharmacokinetic and toxicity studies detailed below. In all cases, the animals were fed standard laboratory food, given free access to water, and housed under 12-h light–dark cycles. All studies were performed according to the tenets of Association for Research in Vision and Ophthalmology for the use of Animals in Ophthalmic and Vision Research and were approved by the animal welfare committee at Hospital de Pediatría JP Garrahan, Argentina (Protocol N°869).

2.8.1. Ocular pharmacokinetics of TPT

TPT disposition in the vitreous after TPT-hydrogel injection was characterized using two methods. First, 6 eyes (3 animals) were assigned to characterize the early times (0–8 h) of TPT release using microdialysis as a multiple-sampling technique allowing us to reduce the number of animals required for study [21]. Then, in a second group of animals (n = 4) 8 treated eyes were enucleated at 24, 48, and 72 h after the injection and immediately frozen to allow vitreous separation from the injected hydrogel for TPT HPLC analysis. In all cases, the animals received an intravitreal injection of 50 μL of TPT-hydrogel into both eyes using a 30G 5/16" gauge needle (BD® insulin syringe, catalog # 328325) as previously described elsewhere [19].

The animals used for the microdialysis study received an intramuscular injection of 30 mg/kg ketamine (Inducmina®, Dr Gray, Argentina), 0.4 mg/kg midazolam (Midazolam Gemepe®, GEMEPE, Argentina) and 1 mg/kg atropine (Norgreen S.A, Argentina) for sedation. General anesthesia was then induced with an intravenous injection of 2.2 mg/kg propofol (Dubernard®, Northia, Argentina) and maintained under mechanical ventilation with isoflurane 2% (Baxter Healthcare, Puerto Rico), 2 μg/kg fentanyl (Fentanilo, Northia, Argentina), and 0.1 mg/kg pancuronium (Bemicin, Northia, Argentina) throughout the study. Before hydrogel injection, the microdialysis probe was inserted into the vitreous space through an incision made with a 25-gauge needle and fixed to the animal conjunctiva. The perfusion fluid (phosphate-buffered saline, pH 7.4) was delivered at a flow rate of 1 μL/min (pump: Kdscientific, LEGATO 101). Subsequently, the hydrogel was injected and dialysates from the vitreous humor were collected at 30-min intervals over a period of 4 h after drug administration. In all cases, *in vitro* recovery was determined before each use by perfusing the probe with a concentrated TPT solution (200 ng/mL) and estimating the recovery with the retrodialysis method [22]. The mean recovery value obtained for the probes was 18.0% (SD: 4.1) and was used to calculate the TPT concentrations in each dialysate sample. At the end of the study all animals were euthanized and eyes were removed for analysis of the TPT concentration in the vitreous. Vitreous samples were treated with acidic methanol (methanol/chlorhidric acid, 10:1) and stored at −20 °C until HPLC analysis.

2.8.2. *In vivo* ocular and systemic safety evaluation of hydrogels

A total of nine rabbits were included in this cohort and assigned to groups A, B, and C (n = 3 in each group). Before intravitreal injections pupillary mydriasis was induced as previously described and 0.5% sterile proparacaine hydrochloride ophthalmic solution (Anestalcon, Alcon Laboratories, Buenos Aires, Argentina) was administered to both eyes for corneal anesthesia [19]. Animals in groups B and C received an intravitreal injection of 50 μL TPT hydrogel at a low and high concentration of the chemotherapeutic agent in the left and right eye, respectively. Group A (control group) received 50 μL of blank hydrogel in the right eye and the left eyes served as controls. Animals were examined before treatment and at week 2 and 4 after the injection. Weight, hair loss, and general condition were evaluated. Complete blood counts were measured by an automated flow cytometer (BC-3000 Plus Auto Hematology Analyzer, Shenzhen Mindray Bio-Medical Electronics Co., Ltd.) [23].

For assessment of ocular toxicity, direct ophthalmoscopic examination was performed at baseline (before hydrogel injection) and 1, 2, and 4 weeks after the injection. Intraocular pressure was measured with a tonometer (Tono-Pen Vet™, Reichert Ophthalmic Instruments, USA) and electrophysiological recordings (ERG) were carried out in both eyes of each anesthetized animal (ketamine hydrochloride, 37.5 mg/kg, IM and xylazine 5 mg/ kg, IM). ERGs were recorded from each eye (Akonic BIO-PC, Akonic, Buenos Aires, Argentina) and the different components including a- and b-wave amplitude and implicit times were evaluated as described elsewhere [7,24].

Table 1
Chemical composition of PCL-PEG-PCL triblock copolymers synthetized by ring opening polymerization.

PCL-PEG-PCL	CL/EO[a]	Mn[a] (g/mol)	Mn[b] (g/mol)	Mw[b] (g/mol)	CL/EO[a]
5235-4000-5235	1.01	14473	6105	8447	1.38
8040-6000-8040	1.03	22080	6524	9424	1.44
1300-1000-1300	1.00	3602	4317	5131	1.19

[a] Calculated by 1H NMR.
[b] Calculated by GPC.

At week 4, animals were euthanized by an overdose of pentobarbital sodium (40 mg/kg body weight) and sodium diphenylhydantoin (5 mg, Euthanyle®, Brouwer Laboratories, Buenos Aires, Argentina) and the eyes were immediately enucleated. Each eye was fixed in 4% paraformaldehyde in 0.1 M of phosphate buffer (pH 7.4) and stained with hematoxylin and eosin for histological examination.

2.9. Topotecan analytical assay

Total TPT concentrations (carboxylate plus lactone) were determined by HPLC coupled with fluorescence detection according to a method previously validated by our group [7]. Briefly, the analysis was performed with an Agilent HPLC system equipped with an Agilent 1100 liquid chromatography pump and an Agilent fluorescence detector set at an excitation/emission wavelength of 370 nm and 530 nm, respectively. Separation chromatography was performed using a Nova-pack C18 reverse-phase column (150 mm × 3.9 mm i.d., 4 μm particle size; Waters, Milford, United states) coupled to a C18 Phenomenex security guard pre-column. Data acquisition and processing was performed using the Agilent ChemStation software. The lower limit of quantitation was 1 ng/mL and the intra and inter-day precision was less than 7%.

2.10. Statistical analysis

Statistical analysis was performed with GraphPad Prism version 6.01 for Windows, (GraphPad Software, La Jolla California USA, www.graphpad.com). *In vitro* release curves were adjusted to the Michaelis-Menten equation and the f-test was used to compare Km. ANOVA analysis was used to test for differences in the parameters calculated in the toxicity study between animal groups. In all cases, the significance level was set at 0.05. The results are expressed as means ± SD.

3. Results and discussion

3.1. Copolymer synthesis and characterization

In order to obtain a sustained-released hydrogel with TPT, three families of PCL-PEG-PCL amphiphilic triblock were successfully synthesized by the ring opening polymerization reaction of ε-caprolactone initiated by PEG precursors of different molecular weight (PEG1000, PEG4000 and PEG6000). The catalyst employed has been approved by the FDA for use in biomedical devices [25]. Results demonstrated that copolymers exhibited differences in (i) the length of both hydrophilic and hydrophobic blocks and (ii) the average molecular weight of the derivative, while the CL/EO molar ratio remained constant (~1.00) as shown in Table 1. We also observed high monomer conversion since the experimental data demonstrated good concordance with the theoretical composition.

To gain further insight in the chemical composition of the derivatives, a GPC analysis was performed. Data revealed the presence of monomodal molecular weight distributions and low polydispersity (PDI) values (<1.45). Differences between GPC and ^{1}H NMR data could be related to the polystyrene standards employed for the GPC calibration curve.

3.2. Preparation and characterization of hydrogels

In recent years, hydrogels have received considerable attention due to their ability to absorb large amounts of water, maintaining their integrity in aqueous solutions [26]. Furthermore, hydrogels based on hydrophobic and hydrophilic blocks have shown promising applications as potential drug-delivery systems. In this framework, we developed PCL-PEG-PCL-based hydrogels (35% w/w) employing triblock copolymers consisting of a central hydrophilic block (PEG) and lateral hydrophobic blocks (PCL). These formulations were successfully prepared by simple copolymer dispersion in water. In a first step, the hydrogels were loaded with 0.05, 0.1, and 0.2 mg of TPT per gram of hydrogel. To gain further insight in the rheological properties of the hydrogels, we evaluated the viscosity of blank hydrogels. All formulations showed pseudoplastic flow characteristics (Fig. S1). Hydrogel prepared with PEG1000 presented a higher viscosity compared with PEG4000 and PEG6000 at 10 RPM (48,000, 28,000 and 22,000 mPa s, respectively). This behavior may result from similar hydrophobic/hydrophilic balances among the three copolymers, but different molecular weights. Therefore, for PEG1000, the amount of macromolecules used to prepare the hydrogel (35%w/w) is higher than for PEG4000 and PEG6000, favoring the formation of a more organized – and therefore more viscous – structure. On the other hand, the addition of TPT did not influence the non-Newtonian behavior of the hydrogels (data not shown). However, an increment in the TPT cargo of the hydrogels leads to a decrease in their viscosity. For instance, PEG4000 with 0.4 mg TPT/g hydrogel showed a value of dynamic viscosity of 4800 mPa s. This observation may be explained by the presence of mannitol as a pharmaceutical excipient in the commercial formulation (Hycamtin®) which was used to prepare the TPT-loaded hydrogels. A similar behavior was observed by Loughlin et al. [27].

Finally, the resulting formulations passed easily through a 30-gauge needle, a small gauge required for intravitreal injection of chemotherapy in eyes with retinoblastoma. This characteristic is one of the main advantages of the TPT-loaded hydrogels developed.

3.3. *In vitro* TPT release study

In vitro TPT release from different PCL-PEG-PCL hydrogel formulations prepared with 0.05, 0.1, and 0.2 mg TPT/g hydrogel was studied using the dialysis method at 37 °C. The release profile of the different formulations is shown in Fig. 1. More than 60% of TPT was released after 24 h for PEG1000 formulations (mean, SD: TPT 0.05 mg/g: 66%, 2; TPT 0,1 mg/g: 63%, 2; TPT 0.2 mg/g, 69%, 4). However, PEG4000 and PEG6000 release was sustained in time and achieved less than 60% of the payload after 24 h of incubation (mean, SD: PEG4000: TPT 0.05 mg/g, 55%, 3; TPT 0.1 mg/g, 59%, 5; TPT 0.2 mg/g, 56%, 6; PEG6000: TPT 0.05 mg/g, 54%, 5; TPT 0.1 mg/g, 57%, 2; TPT 0.2 mg/g, 58%, 9). After 72 h, we observed that all formulations reached a plateau until the end of the assay. At this time, nearly 75–81% of drug was released from PEG1000 for the three loading levels (mean, SD: TPT 0.05 mg/g, 76%, 3; TPT 0.1 mg/g, 75%, 2; TPT 0.2 mg/g, 81%, 2). However, for PEG4000 and 6000, the TPT amount released was between 64 and 68% and 60–69%, respectively (mean, SD: PEG4000: TPT 0.05 mg/g, 64%, 2; TPT 0.1 mg/g, 68%, 6; TPT 0.2 mg/g, 68%, 4; PEG6000: TPT 0.05 mg/g, 69%, 4; TPT 0.1 mg/g, 71%, 4; TPT 0.2 mg/g, 67%, 6). Moreover, we did not observe significant differences in the rate of TPT release among the different drug loading levels ($p > 0.05$). Taking these results into account, we decided to assay a higher level of drug loading (0.4 mg TPT/g hydrogel) into the copolymer with the lowest release rate and molecular weight to obtain a higher degradation rate (PEG4000). This hydrogel (PEG4000, 0.4 mg TPT/g hydrogel) showed a release profile similar to that of lower TPT loads with a burst effect in the

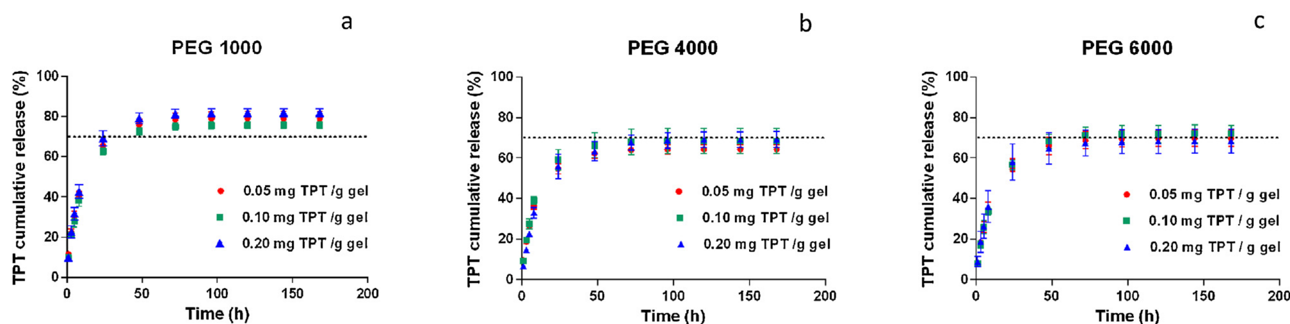

Fig. 1. *In vitro* release of topotecan from the hydrogels.
In vitro accumulated release of topotecan from hydrogels prepared with the three different synthesized copolymers: PCL-PEG-PCL (**A**) 1300-1000-1300, (**B**) 5235-4000-5235, (**C**) 8040-6000-8040. Dotted line shows 70% of accumulated release and the points show individual data.

first 24 h and a sustained release thereafter (Fig. S2). Considering the *in vitro* performance of the developed systems, we decided to perform the *in vivo* tests with the hydrogels prepared with PEG4000 copolymer. For the following *in vivo* experiments we selected two TPT-hydrogels, one as the lowest (to evaluate if a pharmacologically active vitreous concentration is reached) and the other as the maximum (to evaluate the risk of toxicity with higher TPT exposure) TPT concentration that could be delivered into the vitreous of the animals.

Hereinafter, 0.4 mg TPT/g hydrogel is referred to as HLT-hydrogel (HLT, high load TPT) and 0.05 mg TPT/g as LLT-hydrogel (LLT, low load TPT).

3.4. In vitro *cytotoxicity study*

The proliferation of retinoblastoma cells was highly impaired with no signs of proliferation detected after exposing the culture to HLT-hydrogel for 24, 48, 72, or 96 h. In a second set of experiments, after 168 h of TPT release, the remaining HLT-hydrogel still showed high antitumor activity allowing only 36% (SD, 2.6) of cell viability. Interestingly, exposure to blank hydrogel did not affect cell proliferation, which remained the same as in PBS-treated cells. Altogether, the present results indicate that HLT-hydrogel is pharmacologically active against *in vitro* retinoblastoma cells for at least 1 week. Therefore, if translated into the clinics, the injection of the HLT-hydrogel with prolonged therapeutic effect would have an important advantage avoiding repetitive intravitreal injections of active drug in patients.

3.5. In vivo *pharmacokinetic study*

After a thorough *in vitro* characterization, it is important to determine the *in vivo* TPT distribution and elimination using the developed sustained-release formulation. To this end, we carried out a pharmacokinetic study in rabbits and studied the ocular disposition of TPT after intravitreal injection of a single dose of 30 μL of the HLT-hydrogel (loaded with 20 μg of TPT) into the rabbit eye.

As shown in Fig. 2, vitreous TPT data were adequately fitted to a one-compartment model as implemented in ADAPT 5 [28]. The vitreous volume was fixed to 1.7 mL based on previous reports on the vitreous volume of rabbits [21]. The calculated maximum vitreous concentration was 2230 ng/mL and was attained 4 h after the injection. Interestingly, and as expected based on the sustained-release characteristics of the developed formulation, the attained maximum vitreous concentration was lower than that previously reported after an intravitreal injection of a 5 μg/mL aqueous solution of TPT in the same animal species [7]. Thus, a lower but more sustained release of TPT loaded in hydrogel would be advantageous

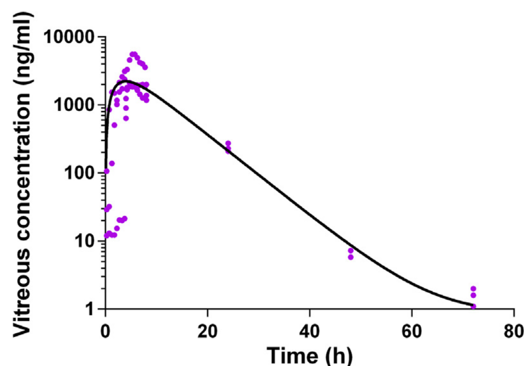

Fig. 2. Topotecan concentration in the vitreous of rabbits after intravitreal injection of HLT-hydrogel.
Full symbols represent individual data points for vitreous concentrations at different times after the injection and the line, the best predicted concentrations.

to avoid high drug exposure levels in the retina and thereby reduce toxicity. In modified release dosage forms it is necessary to avoid a rapid drug release of the entire amount or a significant fraction of the active substance (dose dumping effect [19]) because of the potential toxicity. In the present pharmacokinetic study in rabbits no dose dumping effect was observed in the TPT release from the hydrogel supporting the development of the current platforms.

Other pharmacokinetic parameters calculated showed that the apparent elimination rate constant from the vitreous was 0.398 h^{-1} (0.04) while the mean appearance rate in the vitreous was 0.117 h^{-1} (0.005).

In addition, total TPT concentration in the vitreous of the injected eyes was higher than the inhibitory concentration of commercial retinoblastoma cells reported to be 30 nM (14 ng/mL) [4] for 24 h. Hence, we could expect antitumor activity for at least the first 24 h after the injection. Thereafter, vitreous TPT levels declined and concentration was around 1.6 ng/mL for 72 h.

Another parameter of TPT vitreous humor exposure we calculated was the area under the concentration *versus* time profile (AUC). TPT vitreous AUC was found to be 31,045 ng*h/mL. In a previous study, after an intravitreal injection of 5 μg TPT solution total TPT vitreous AUC was 26.62 μg*h/mL or 26,620 ng*h/mL [19] .Thus, despite a 4-fold increase in the dose of TPT loaded in the hydrogel compared with that injected as an aqueous solution, a similar AUC was obtained up to 72 h after the administration of the hydrogel. Thereafter, the remaining load in the hydrogel would be released in a sustained fashion supporting the use of a sustained-release device for intraocular purposes.

The rabbit is an adequate preclinical model to study human ocular pharmacokinetics [19]. Nonetheless, we need to acknowledge

the limitation of working with a normal animal model to translate the results into the clinics of an eye affected by tumor. The presence of the tumor may disrupt the blood-retina barrier and alter the pharmacokinetics of the studied drug [29]. Moreover, interspecies differences in the anatomy and physiology of the eye may limit the translation of these findings into humans [29].

3.6. Toxicity study

Safety studies were performed in rabbits to assess the potential ocular and systemic toxicity after intravitreal injection of blank hydrogel, LLT-, or HLT-hydrogel. The results showed that the cornea, anterior chamber, lens, and vitreous remained clear in all animals throughout the study period. No fundus changes attributable to TPT or blank hydrogel toxicity were evident in any of the animal groups. The intraocular pressures remained within the normotensive range for all treated eyes. This is an important observation as an increase in the intraocular pressure may be associated with uveitis and secondary hypertension [29].

Systemic toxicity was evaluated as changes in animal behavior, weight loss, or changes in pathological biochemical parameter values. We observed a temporal variation of body weight for the blank and LLT hydrogels showing weight gain over the study period ($p < 0.05$). In the case of the HLT hydrogel the temporal variation of body weight did not show significant differences implying that the weight of the animals remained within the same along the studied period ($p > 0.05$). This was an expected and promising finding as animals increase body weight under normal conditions. Hence, blank or TPT-loaded hydrogels allowed for normal feeding behavior and no hair loss of the treated animals.

Hematologic values recorded during the follow-up period for the three groups showed no significant differences in any of the evaluated parameters at any period of time compared to the values obtained before the injection of the hydrogels (Table 2, $p > 0.05$). Thus, no significant change in the evaluated parameters could be detected as a result of blank or TPT-loaded hydrogel injection. Although TPT is a well-known myelosuppressive agent [5], TPT-loaded hydrogels did not induce bone marrow toxicity in our animal model probably due to the slow release of the chemotherapeutic agent from the hydrogel and low systemic exposure.

In the cohort of animals used for the assessment of ocular toxicity, no significant changes were found in the ERG parameters between the non-treated (control) eyes and the eyes injected with blank hydrogel in the same animals of Group A ($p > 0.05$). Therefore, blank hydrogels were safe to the retina. In addition, ERG recordings were normal in all LLT- and HLT-hydrogel-treated eyes with little or no change in a- and b-waves and implicit times as shown in Table 3. Thus, increasing the loading dose of TPT in the hydrogel from 2.5 μg to 20 μg (0.05 and 0.4 mg TPT per gram of gel injected into Group B and C, respectively) did not lead to ocular toxicity but allowed us to increase the total dose almost 10 times. As shown in Table 3, eyes showed similar amplitude and implicit time values among all groups without statistical differences when comparing parameters between groups or before any injection and at different times during the study period ($p > 0.05$).

Light microscopic examinations revealed no histologic evidence of retinal damage induced by the blank or TPT-loaded hydrogel at either loading dose studied. Representative micrographs of retinal sections from eyes of rabbits of groups A, B, and C are shown in Fig. 3**a**–**c**. The inner and outer nuclear layers of the retina, the photoreceptor structures, and ganglion cells remained normal in eyes treated with blank hydrogels and both TPT doses. No differences in the retinal thickness were observed between blank and TPT-loaded hydrogel treated eyes or compared to control eyes. Nonetheless, we observed macrophage infiltration in A, B, and C eyes treated with blank, LLT, and HLT-hydrogels, respectively, as a potential inflam-

Table 2
Clinical and biochemical characteristics after intravitreal injection of blank, LLT, or HLT hydrogels in rabbits.

	Animal Group		
	Group A	Group B	Group C
Weight (kg)			
Before treatment	1.9 (1.9–2.0)	1.9 (1.6–2.3)	2.2 (1.5–2.3)
1st week	2.0 (2.0–2.0)	2.0 (1.6–2.3)	2.0 (1.6–2.2)
2nd week	2.1 (2.1–2.2)	2.2 (1.8–2.4)	2.2 (1.8–2.3)
4th week	2.2 (2.2–2.4)	2.4 (1.9–2.5)	2.3 (2.1–2.5)
WBC (1000/mm^3)			
Before treatment	11.6 (7.9–12.3)	12.5 (7.7–13.2)	7.6 (7.3–9.8)
2nd week	8.6 (8.3–9.9)	8.1 (8.1–15.9)	7.6 (6.8–8.5)
4th week	9.0 (9.0–11.1)	7.7 (6.5–14.2)	7.8 (6.5–8.7)
Neutrophils (%)			
Before treatment	7.7 (3.9–8.0)	8.3 (2.8–9.3)	5.1 (3.4–5.5)
2nd week	3.8 (2.7–4.3)	3.2 (3.0–9.4)	3.3 (2.7–3.9)
4th week	5.4 (5.0–7.0)	3.7 (2.0–8.4)	4.5 (2.6–5.1)
Hematocrit (%)			
Before treatment	38 (37–38)	37 (35–37)	37(37–38)
2nd week	44 (43–45)	38(35–40)	39.0 (32–41)
4th week	42 (40–43)	38 (37–39)	42 (38–43)
Platelets (*1000/mm^3)			
Before treatment	480 (206–482)	480 (206–482)	480 (206–482)
2nd week	451 (361–455)	444 (313–448)	415 (314–419)
4th week	422 (288–450)	366 (281–409)	405 (281–448)
Hemoglobin (g%)			
Before treatment	12.3(11.3–12.7)	12.0(10.9–12.6)	13.7(12.7–13.7)
2nd week	14.0(13.3–14.5)	12.0(10.9–12.6)	12.2(10.2–12.5)
4th week	13.4(13.2–13.7)	12.5(11.8–13.6)	13.6(12.0–13.7)
Red blood cells ($10 \times ^6$/mm^3)			
Before treatment	6.2 (5.6–7.2)	5.9 (5.3–6.2)	5.9 (5.4–6.6)
2nd week	6.3 (6.1–6.6)	5.8 (5.6–5.9)	6.0 (4.3–6.2)
4th week	6.3 (5.8–6.3)	6.0 (5.7–6.7)	6.5 (5.1–6.7)

Data are shown as median (range). Group A, B, and C correspond to animals treated with blank, LLT, and HLT hydrogels, respectively.

Table 3
Electroretinography response after intravitreal injection of blank or TPT loaded hydrogels.

	Animal Group		
	Group A	Group B	Group C
b-wave			
Amplitude			
Before treatment	167 (13)	158 (32)	189 (38)
1st week	171 (38)	138 (78)	199 (28)
2nd week	141 (67)	158 (43)	154 (25)
4th week	166 (41)	157 (14)	134 (11)
Implicit time			
Before treatment	36 (1)	37 (1)	36.3 (0.6)
1st week	37 (1)	37 (0)	36.1 (0.2)
2nd week	35 (5)	38 (0)	34 (5)
4th week	36 (1)	37 (1)	36.3 (0.6)
a-wave			
Amplitude			
Before treatment	62 (8)	89 (9)	89 (10)
1st week	73 (16)	73 (23)	95 (8)
2nd week	56 (24)	93 (20)	79 (11)
4th week	74 (3)	82 (11)	60 (22)
Implicit time			
Before treatment	13.2 (0.4)	12.8 (0.4)	13.0 (0.5)
1st week	12.7 (0.6)	13.3 (0.8)	12.3 (0.5)
2nd week	13.0 (0.6)	13.5 (0.2)	12.5 (0.6)
4th week	13.1 (0.2)	13.2 (0.4)	14.0 (2.0)

Data are presented as mean (SD). Group A, B, and C correspond to animals treated with blank, LLT, and HLT hydrogels, respectively. 3 animals were studied in each group.

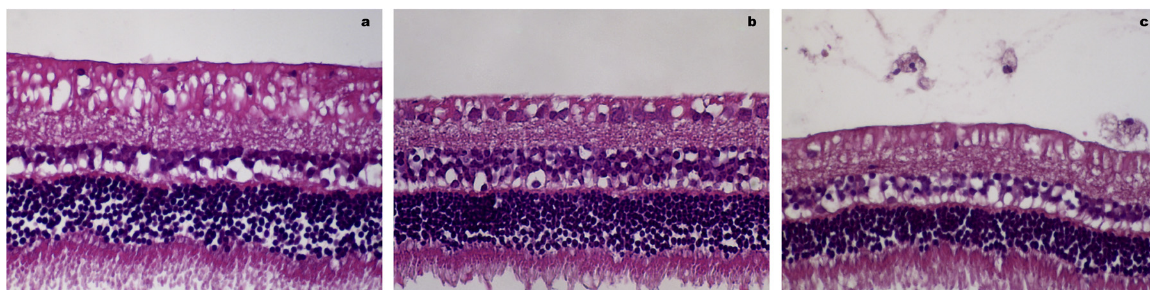

Fig. 3. Retinal sections of eyes injected with (**a**) blank, (**b**) LLT, or (**c**) HLT hydrogel.

matory response. Although this finding was expected as an ocular response to the intravitreal injection of a foreign substance, it is of particular interest as pro-inflammatory cytokines produced by macrophages play an important role in inflammatory, neovascular, and neurodegenerative processes [30]. It should be further assessed if the presence of infiltrating macrophages in the vitreous as a result of hydrogel injection may trigger neovascularization, ocular inflammation, or a poor prognosis for retinal function. Finally, repetitive intravitreal injections of aqueous solutions of topotecan may lead to different complications including endophthlamitis, retinal detachment, cataracts and even extraocular seeding of tumor cells. These complications may be avoided by the single intravitreal injection of the topotecan hydrogel developed in the present study. It has to be acknowledged that the injected hydrogel may lead to patient discomfort and blurred vision as a result of the *in vivo* degradation of the hydrogel in the vitreous cavity though not observed in our animal model. Nonetheless, translation into the clinics should be performed with caution.

4. Conclusions

In this study PCL-PEG-PCL copolymers were successfully synthesized and employed to prepare TPT-loaded (0.05, 0.1, 0.2, and 0.4 mg TPT per gram of hydrogel) sustained-release hydrogels. The hydrogels showed a sustained release of TPT *in vitro* for at least one week. TPT-loaded hydrogel was cytotoxic to retinoblastoma cells for at least one week supporting the hypothesis of prolonged activity of the chemotherapeutic agent loaded in the hydrogel. In rabbits, neither blank nor TPT-loaded hydrogels induced myelosuppression, weight loss, or any other pathological changes. Moreover, blank or TPT-loaded hydrogels were safe for the eyes without triggering retinal toxicity according to the ERG responses or histopathological assessment.

Altogether, the developed hydrogels loaded with TPT may be useful as an additional strategy for the treatment of retinoblastoma.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2016.07.001.

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