NOVEL BIODEGRADABLE POLYESTERAMIDE MICROSPHERES FOR CONTROLLED DRUG DELIVERY IN OPHTHALMOLOGY

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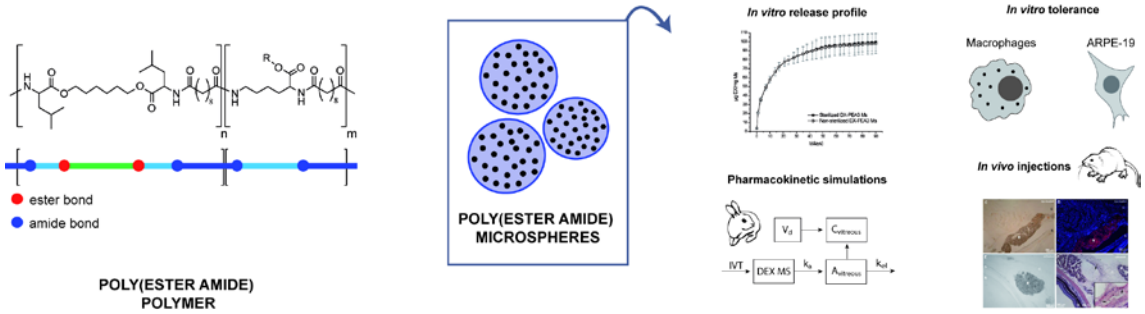
ABSTRACT

Most of the posterior segment diseases are chronic and multifactorial and require long-term intraocular medication. Conventional treatments of these pathologies consist of successive intraocular injections, which are associated with adverse effects. Successful therapy requires the development of new drug delivery systems able to release the active substance for a long term with a single administration. The present work involves the description of a new generation of microspheres based on poly (ester amide)s (PEA), which are novel polymers with improved biodegradability, processability and good thermal and mechanical properties. We report on the preparation of the PEA polymer, PEA microspheres (PEA Ms) and their characterization. PEA Ms (~15 µm) were loaded with a lipophilic drug (dexamethasone) (181.0 ± 2.4 µg DX/mg Ms). The *in vitro* release profile of the drug showed a constant delivery for at least 90 days. Based on the data from a performed in *vitro* release study, a kinetic ocular model to predict *in vivo* drug concentrations in a rabbit vitreous was built. According to the pharmacokinetic simulations, intravitreal injection of dexamethasone loaded PEA microspheres would provide release of the drug in rabbit eyes up to 6 months. Cytotoxicity studies in macrophages and retinal pigment epithelial cells revealed a good *in vitro* tolerance of the microsystems. After sterilization, PEA Ms were administered *in vivo by* subtenon and intravitreal injections in male Sprague-Dawley rats and the location of the microspheres in rat eyes was monitored. We conclude that PEA Ms provide an alternative delivery system for controlling the delivery of drugs to the eye, allowing a novel generation of microsphere design.

GRAPHICAL ABSTRACT



KEYWORDS Ocular drug delivery; microspheres; poly(ester amide); tolerance; dexamethasone; intraocular injection.

INTRODUCTION

Most of diseases affecting the posterior segment of the eye are related with visual impairment and blindness. The effective treatment of these pathologies is one of the major challenges in drug delivery as most of them are chronic and multifactorial. Among them, aged-related macular degeneration, diabetic retinopathies and glaucoma produce irreversible visual damage and blindness [1]. These diseases are becoming more and more prevalent in the aging populations, and nowadays tens of millions of patients are affected worldwide. Depending on the disease, the medications should be delivered to the retinal cells, retinal pigment epithelium or choroid. Furthermore, therapeutic concentrations of the active substance in the intraocular target site have to be maintained during a long period of time.

Due to the ocular barriers, it is difficult to deliver effective drug concentrations to the posterior tissues of the eye using non-invasive routes such as topical or systemic administration [2]. It is well known that after topical administration only very low drug concentrations are reached in the retina and choroid [3]. This is due to the obstacles of drug penetration that include the short residence time of formulations on the ocular surface, the presence of tissue barriers (cornea, lens, conjunctiva, sclera), and flow mediated drug loss factors (conjunctival blood flow, aqueous humor flow) that limit the drug access to the retina and choroid. Although systemic administration is used to deliver some drugs to the eye (e.g. corticosteroids), this route is restricted by the systemic toxicity of the drugs and reduced access to the target site, mainly due to the blood-aqueous and blood-retinal barriers [2].

The most effective method of drug delivery to the back of the eye is through intraocular administrations, mainly intravitreal injections. However, intravitreal administration is an invasive mode of drug delivery and it is sometimes associated with adverse effects (endophthalmitis, hemorrhages, damage of lens or retinal detachment) and it requires frequent visits of the patients to the clinics. Besides, most low molecular weight drugs have short intravitreal half-lives (2-10 hrs), so they have to be administered frequently to be clinically feasible. Controlled drug delivery systems, such as nano- and microcarriers, as well as implants, able to release and maintain effective active substance levels over long periods of time, would prolong the dosing interval to months [4, 5]. Biodegradable micro- and nanoparticulate systems are emerging therapeutic tools as they can be administered as a conventional injection by periocular (subconjunctival, subtenon, juxtascleral) and intraocular routes and they are cleared from the site of administration over time.

Ophthalmic drug delivery systems can be made with a variety of biodegradable materials such as polyesters (lactide and glycolide copolymers, polycaprolactones, poly(β-hydroxybutyrates)), polyamides (including natural polymers such as collagen, gelatin and albumin), heteropolysaccharides (chitosan) or lactic and glycolic acid polymers and copolymers, among

others. Poly-lactic-co-glycolic acid (PLGA) has been widely used for the development of a number of drug delivery systems, such as the intraocular commercialized implant loaded with dexamethasone (Ozurdex®). The advantages of biodegradable implants over the non-erodible devices in the clinical practice have promoted the interest in novel polymers adequate for intraocular drug delivery purposes.

We have studied a new generation of microspheres based on poly(ester amide)s (PEA). The PEAs are amino acid containing biodegradable polymers combining ester and amide groups in the polymer chain. This chemical structure contributes to improved biodegradability, processability and mechanical properties of the materials (via intra- or inter-chain hydrogen bonding interactions though its amide groups). Furthermore, an important polymer feature is that the current composition of PEA predominantly degrades through surface erosion mechanism [6]. These materials have already demonstrated good biocompatibility showing little or no inflammation both *in vitro* and *in vivo* [7] including in an ophthalmic setting [8]. Extruded PEA fibrils have been implanted in both periocular (subconjunctival) and intravitreal routes in a rabbit experimental model. Readouts after 1, 3, 5 days and 2, 4, 8 weeks have shown excellent material tolerance and tissue biocompatibility. Further work [9] investigated blood and cellular *in vitro* responses of PEA. The findings of the study revealed that monocytes adherent to PEA secreted reduced levels of the pro-inflammatory interleukins (IL)-6 and IL-1β into the culture supernatant relative to those on comparative polymers but secreted significantly higher amounts of the anti-inflammatory mediator, IL-1 receptor antagonist. A PEA coating on cardiovascular stent has been reported in a phase III, 2-armed clinical study [10]. The 24-months follow up of this study reports absence of Major Adverse Cardiac Events (MACE) and suggests that the tested PEA-coated stent is safe [11].

The present work is focused on the study of the ability of biodegradable polyesteramide to form microspheres for ophthalmic drug delivery purposes. Microspheres present several advantages among other ophthalmic drug delivery forms for different reasons: (a) Drugs encapsulated in microspheres are protected from degradation and physiological clearance, (b) the release kinetics of the drug can be adjusted by varying the technological parameters of these systems and (c) microspheres can be injected as a suspension using conventional needles (27-34G) without surgery [12].

This study shows the synthesis of polyesteramide (PEA) polymers and the preparation, sterilization, “in vitro tolerance” and delivery characteristics of microspheres (Ms) made from PEA and loaded with a lipophilic drug model (dexamethasone). After studying the impact of gamma sterilization on the properties of these systems, we have built a kinetic ocular model with *in vitro* release data to predict *in vivo* drug concentrations in a rabbit vitreous model. Then, we studied the impact of the different reagents, solutions and processes required to perform histological procedures, on the properties of Ms, in order to establish the most appropriate

inclusion techniques for *in vivo* studies. Finally, we have analyzed the behavior of PEA Ms after injection in the subtenon space and in the vitreous humor of rats with the aim of determining whether or not a satisfactory amount of PEA Ms were placed in the desired locations.

MATERIAL AND METHODS

Material

Polyvinyl alcohol 67 kDa (PVA) was provided by Merck (Darmstadt, Germany). Acetonitrile (ACN), dichloromethane (DCM) and methanol (MET) were purchased from Sigma-Aldrich (Schenelldorf, Germany). Tetrahydrofuran (THF) was supplied by Teknokroma (Barcelona, Spain). Super gradient acetonitrile (ACN) was purchased from Lab-Scan (Madrid, Spain). Dexamethasone (DX), dimethyl sulphoxide (DMSO) and 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Schnelldorf, Germany). Freshly produced MilliQ water (W) was used in all the experiments. Poly-(D,L-lactide-co-glycolide) PLGA ratio 50:50 (35 kDa; Resomer 503) was purchased from Boehringer Ingelheim GmbH (Ingelheim am Rhein, Germany). Polyvinyl alcohol 72 kDa (PVA) and anhydrous DMF were obtained by Merck KGaA (Darmstadt, Germany). Polyethyleneimine (PEI) microspheres were supplied by Micromod (Rostok, Germany). Unless noted otherwise, cell culture reagents were provided by Life Technologies (Carlsbad, CA, USA). Macrophages (RAW 264.7) and human retinal pigment epithelial cell lines (ARPE-19) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Synthesis of polyesteramide copolymers

The polymer in this study is a biodegradable poly(ester amide) based on α-amino acids, aliphatic dicarboxylic acids and aliphatic α-ω diols. Among this class of materials the AA-BB hetero-chain polymers offer the greatest versatility in terms of molecular level design to tailor drug release properties. The selected PEA is depicted on Fig. 1 and it comprises three type of building blocks randomly distributed along the polymer chain.

Figure 1 Structure of PEA III Ac Bz.

The polymer was synthesized according to a procedure reported previously [13]. Briefly, the polymer was prepared via solution polycondensation of di-p-toluenesulfonic acid salts of bis-(α-amino acid) α,ω-diol diesters, lysine benzyl ester and di-N-hydroxysuccinimide sebacate in anhydrous DMF. The use of pre-activated acid in the reaction allows polymerization at low

temperature (65^0C) affording side-product free polycondensates and predictable degradation products [14]. The polymer was isolated from the reaction mixture in two precipitation steps. The polymer was characterized by ^{1}H NMR spectroscopy and THF based GPC relative to polystyrene standards.

Polymer characterization

^{1}H NMR spectra were obtained on a Bruker Avance 500 MHz Ultrashield NMR; samples were recorded in ethanol d_6.

Molecular weight and molecular weight distributions of PEA were determined by GPC equipped with RI detector. Samples were dissolved in THF at a concentration of approximately 5 mg/mL and were run at a flow rate of 1 mL/min at 50 ºC. The molecular weights were calibrated to a narrow polystyrene standard calibration curve, using Waters Empower software.

Preparation of microspheres

PEA microspheres (PEA Ms) were prepared via the emulsion solvent-evaporation technique. Briefly, 2 mL of an organic phase composed of PEA/DCM (15%) was emulsified with 5 mL of an aqueous solution of PVA (2%) at 8,500 rpm for 1 min (Polytron PT 3000, Kinematica, Lucerna, Switzerland). This emulsion was subsequently poured onto 100 mL of an aqueous solution of PVA (0.1%) and kept under constant stirring for 4 hours, to allow organic solvent evaporation and hardening of microspheres. After this process, PEA Ms were washed and filtered at low temperature (4ºC). After that, microspheres were suspended in 1 mL of an aqueous solution of mannitol (2%), used as cryoprotectant. The resulting suspension was freeze-dried. Ms were kept at 4ºC in desiccators until use. To prepare dexamethasone-loaded PEA microspheres (DX-PEA Ms), 60 mg of DX were dispersed by gentle sonication (Sonicator XL, Head Systems, Iowa, USA) in 2 mL of the organic phase composed of PEA/DCM (15% w/v), for 3 minutes. Then, microspheres were prepared following the same steps described above. All the procedure was performed protecting dexamethasone from exposure to light. The percentage yield of each batch was calculated.

To help identify the location of the microspheres in ocular tissues after in vivo injections, a PEA polymer that contained 0.02 % w/w chromoionophore II (absorption: 520-600nm, emission: 660nm Fluka, Sigma-Aldrich, Schnelldorf, Germany) was employed. The dye molecule was incorporated to the polymer by dissolving of a calculated amount of chromoionophore II in 10 % polymer solution in ethanol. Later the solvent was evaporated and obtained material was used for preparation of microspheres. For the *in vitro* tolerance studies, microspheres composed of PLGA were used as reference of a non-toxic polymeric material. Both, fluorescent PEA Ms and

PLGA Ms, were prepared with techniques based on the emulsion-solvent evaporation method described above.

Quantification of dexamethasone

Dexamethasone was quantified by high performance liquid chromatography (HPLC) using a liquid chromatograph with a pump M520, a UV detector M490E, an autosampler 712D WTSP and the Empower Login HPLC System Manager Software, all by Waters (MA, USA). The chromatographic separation was achieved with a C18 column Tracer Excel 120 ODSA (particle size 5 µm, 150 mm x 4mm; Teknokroma, Barcelona, Spain). The mobile phase flow was set at 1 mL/min and the injection volume was 20 µL. The absorbance of the eluent was monitored at 254 nm. All the analyses were performed at 45 ± 0.5ºC.

The composition of the mobile phase A was methanol:ACN:water (3:3:4). The mobile phase B was composed of 100% ACN. Both A and B were vacuum-filtered through 0.45 µm nylon millipore membrane (Merck, Darmstadt, Germany), and degassed by ultrasonication for 15 minutes before use (Elma Transsonic 460, Singen, Germany). A gradient elution method was employed and the chromatograph was programmed as follows: 100% A for 10 min, followed by 0 to 100% B over 15 min, then 100 to 0% B over 5 min and finally 100% A over 5 min. The HPLC method was validated with respect to linearity, accuracy and reliability in the range of concentrations of 2-20 µg/mL.

Microsphere characterization

Microspheres' morphology was evaluated by scanning electron microscopy (SEM; Jeol JSM-6335F, Tokyo, Japan). Before examination, samples were sputter coated with gold.

Mean particle size and particle size distribution were measured by light scattering in a Microtrac S3500 Series Particle Size Analyzer (Montgomeryville, PA, USA). Samples were analyzed by dispersing the microspheres in MilliQ Water. Data are presented as mean volume diameter ± standard deviation of three independent measurements.

To determine the encapsulation efficiency of DX, 5 mg of solid DX-PEA Ms were dissolved with 200 µL DCM. Then, 800 µL of ACN were added and, after strong mixing by vortex for 2 min, the solution was centrifuged at 15,000 rpm (4ºC, 15 min). The supernatant was further removed, filtered (0.45 µm membrane) and analyzed by HPLC, following the method described above. The entrapment efficiency was determined by using the formula: drug entrapment efficiency = (experimental drug content/theoretical drug content) x 100.

The chemical stability of PEA during the microsphere fabrication process was evaluated by high-performance gel permeation chromatography (GPC). To this, microspheres were dissolved in THF (1 mg/mL). Then, samples were filtered with a PTFE membrane (0.2 µm). Two columns PGgel 3 µm MIXED-E and PGgel 5 µm MIXED-D, both of 7.8 mm x 300 mm (Varian, Polymer Laboratories, Church Stretton, UK), were connected in series to increase the accuracy of the procedure. The equipment consisted of a Waters 1525 binary HPLC pump and a Waters 2414 Refractive Index Detector (Waters, Saint-Quentin en Yvelines, France). The flow rate was set to 1 mL/min of THF, and temperature of the process was 33ºC. Prior to measurements, the GPC columns were calibrated with polystyrene standards of molar masses: 381, 1100, 2950, 6520, 18,600 and 43,700 g/mol, supplied by Waters (Mainz, Germany) and 10,100, 24,600 and 72,450 g/mol, purchased from Varian (Polymer Laboratories, Church Stretton, Shrosphire, UK).

Sterilization of microspheres

Microspheres were sealed in glass vials and irradiated with ^{60}Co (Gamma Sterilization Unit of Aragogamma S.A., Barcelona, Spain) at low temperature (-80ºC). Following the USP recommendations, a dose of 25 kGy was applied to ensure an effective sterilization [15]. Sterilized formulations were characterized as described above.

In vitro release studies

Solid DX-PEA Ms (5 mg) were incubated in 1.5 mL of a phosphate buffered solution isotonized with sodium chloride (PBS, pH 7.4; with KH_2PO_4 1.54 mM, Na_2HPO_4-$7H_2O$ 2.71 mM and NaCl 155.17 mM). Samples were placed in a shaker at a constant agitation speed of 100 rpm (NE5 Shaking Water Batch, Nikel Electro Ltd., Weston-super-Mare, United Kingdom) at 37ºC. At pre-set times (1 h, 24 h and twice every week during 90 days) the supernatant was recovered and replaced with the same volume of fresh PBS. Supernatant was analyzed by HPLC to determine the amount of released DX. Studies were performed under sink conditions and in triplicate. Microsphere' morphology was analyzed with SEM during the first two weeks. Sterilized and non-sterilized DX-loaded microspheres' release profiles were compared with the evaluation of the similarity factor f_2 [16].

Kinetic modeling of dexamethasone release from microspheres

A kinetic ocular model was built, in order to predict dexamethasone concentrations in the rabbit vitreous during drug delivery from the PEA microspheres. The simulations were carried out with Stella software (ISEE systems 10.0). Firstly, the *in vitro* release data of dexamethasone was used to define the release rate of dexamethasone from the microspheres using curve fitting. This represents drug release in sink conditions ($y = M0 + M1 \log(x)$, where x = time and y = released dexamethasone (%). M0 = 18.19 and M1 = 18.5 ($R^2 = 0.9425$).

Secondly, the model for *in vivo* delivery of dexamethasone was built (Fig. 2) and different dexamethasone doses (100-1000 μg) were used to simulate drug concentration in the vitreous after microsphere administration.

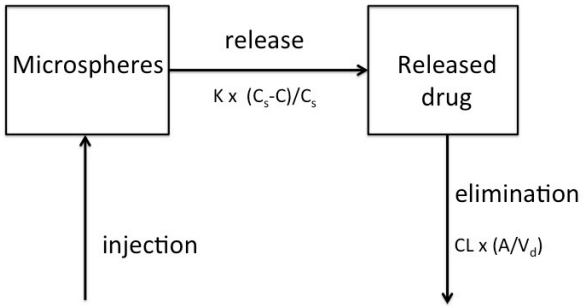


Figure 2 Ocular model of vitreal dexamethasone after its release from the PEA microspheres. A: released dexamethasone amount in the vitreous, V_d: volume of distribution, CL_{ivt}: clearance of dexamethasone from the vitreous.

Drug release was defined as k = (release rate in vitro * (C_s – $C_{vitreous}$) / C_s), where C_s = dexamethasone solubility in water (0.089 mg/mL at 25 °C, Drug Bank) and $C_{vitreous}$ = free drug concentration in the vitreous (μg/mL). Dexamethasone concentrations in the vitreous were simulated using the aforementioned formulation parameters and pharmacokinetic parameters of dexamethasone in the rabbit eyes (CL_{ivt} = 0.668 ml h^{-1}, volume of distribution, V_d = 1.5 mL) [17]. V_d was estimated based on the previous report that demonstrates narrow range of intravitreal V_d values of ocular drugs [29]. CL_{ivt} of dexamethasone was calculated using the QSPR model for intravitreal drug clearance in the rabbit vitreous [29]. The calculation is as follows: $LogCL_{ivt}$= - 0.25269 - 0.53747 (LogHD) + 0.05189 ($LogD_{7.4}$). Dexamethasone is within the applicable chemical space of the model (supplementary material).

In vitro tolerance of microspheres

Cytotoxicity studies were performed with two cell lines: mouse macrophages (RAW 264.7) and retinal pigment epithelial cells (ARPE-19). *In vitro* citotoxicity was assessed using mitochondrial-dependent reduction of the tetrazolium salt, 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan.

RAW 264.7 cells were maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 2% penicillin/streptomycyn, and placed onto 24-well plates (9.5 x 10^4 cells/well). After an incubation period of 4 hours (37ºC and 5% CO_2), the media was changed to RPMI supplemented with 2% FBS, and cells were exposed to microspheres dispersed in DPBS for 20 hours. Then, the medium was carefully removed and the cells reacted

with MTT (5 mg/mL in PBS) for 3 hours at 37ºC. The reaction product, formazan, was extracted with DMSO. The extent of the reduction of MTT to formazan within cells was quantified by the measurement of optical density at 550 nm, using a microplate reader (model 6010152EU; DigiScan, Eugendorf, Austria). Microspheres' dispersions were tested in the range 0.001-2 mg/mL. PEI microspheres (8 µm size) were used as positive control and PLGA microspheres (20 µm size) as reference of a non-toxic material. Microspheres were dispersed with HBSS (without Ca^{2+} or Mg^{2+}) in all cases. Assays were performed in triplicate and results are expressed as percent of reduction in cell viability compared to vehicle-treated cells for at least three independent experiments.

Human retinal pigment epithelial cells (ARPE-19) were cultured in a Dulbecco's modified Eagle's medium (DMEM): Nutrient Mixture F12, 1:1 mixture, supplemented with 10% heat inactivated FBS, 2 mM L-Glutamine, 50 U/mL streptomycin and 50 U/mL penicillin. The cells were maintained at 37 ºC in a humidified atmosphere containing 7% CO_2. For the cytotoxicity assay, ARPE-19 cells were seeded on a 24-well plate one day prior to the experiment day at a density of 80 000 cells/well. On the day of experiment, PEA microspheres and PLGA microspheres in HBSS buffer (without Ca^{2+} or Mg^{2+}) were incubated with the ARPE-19 cells, at a concentration of 0.1 – 2 mg/mL. Poly-L-lysine (15-30 kDa, Sigma-Aldrich, MO, USA) in DPBS buffer (without Ca^{2+} or Mg^{2+}) was used as a positive reference polymer in the assay, at a concentration of 0.001 – 2 mg/mL. After 5 hour of incubation, the wells were washed and replaced with supplemented growth medium. On the third day, 24 hours later, MTT in serum-free growth medium was added to the cells (0.5 mg/mL). MTT is converted to a water-insoluble formazan by mitochondrial activity of living cells. The formazan crystals were solubilised with 10% sodium dodecyl sulphate (Bio-Rad, Ca, USA) - hydrochloric acid and on the fourth day the optical density was measured at 570 nm (VarioSkan Flash, Thermo Scientific, Madrid, Spain). The percentage cell viability was calculated by comparing the viability of the PEA and PLGA microspheres or PLL treated cells with HBSS or DPBS treated cells, respectively. Cytotoxicity data was obtained from at least three different experiments or more, by testing triplicate wells per sample.

Handling of microspheres for in vivo injections

Unloaded PEA Ms and dye-loaded PEA Ms suspended in PBS were analyzed under both, light and fluorescence optic microscopy. In order to ascertain the most appropriate techniques for in vivo biocompatibility assessment, solid PEA Ms were exposed to reagents (4% paraformaldehyde, 2% glutaraldehyde, xylene, EtOH and acetone), solutions (distilled water and PBS) and processes (freezing at -20ºC and heating at 60ºC) required to perform histological procedures, specifically paraffin and epoxy resins embedding and tissue frozen. For that, 100 µL of the reagents and solutions were added to the PEA Ms powder and kept in contact with them from 5 minutes to 5 hours.

In vivo injections

Animals and anaesthetics

Rats were treated in accordance with the Spanish Laws and the Guidelines for Human Endpoints for Animals Used in Biomedical Research. This study was approved by the Ethics Committee for Animal Research of Complutense University of Madrid. Also, animal manipulations followed institutional guidelines, European Union regulations for the use of animals in research, and the ARVO (Association for Research in Vision and Ophthalmology) statement for the use of animals in ophthalmic and vision research.

The experiments were performed on adult male Sprague-Dawley rats (250 g) obtained from Harlan Laboratories (Udine, Italy). The animals were housed in temperature- and light-controlled rooms with a 12h light/dark cycle and *ad libitum* access to food and water. Light intensity within the cages ranged from 9 to 24 lux. All surgical procedures were performed under general anaesthesia induced with an intraperitoneal (i.p.) injection of a mixture of Ketamine (75 mg/kg, Ketolar®, Parke-Davies, S.L., Barcelona, Spain) and Xylazine (10 mg/kg, Rompún®, Bayer, S.A., Barcelona, Spain). During recovery from anaesthesia, rats were placed in their cages, and an ointment containing tobramycin (Tobrex®; Alcon S.A., Barcelona, Spain) was applied on the cornea to prevent corneal desiccation and infection. Additional measures were taken to minimize discomfort and pain after ocular injection.

Intraocular injection

To ensure appropriate and reproducible intraocular injection of the PEA Ms, freshly prepared microsphere suspensions in Balanced Salt Solution (BSS; Alcon, TX, USA) were released through different gauge needles (25G, 27G, 30G and 32G). Microspheres were quantified in a Neubauer chamber cell counting (BLAUBRAND® counting chambers, Germany) by using the manual counting tool of the Metamorph Imaging System. To analyse whether time elapsed between PEA Ms suspension preparation and injection influences microsphere behaviour, the Neaubauer cell counting chamber was loaded with PEA Ms suspensions by using a 30G needle 5, 20, 30, 60, 90, 120 and 180 minutes after microsphere suspension preparation.

In order to analyze PEA Ms behavior *in vivo* and to ascertain that a satisfactory amount of PEA Ms were placed in the desired location, rat eyes were injected periocularly (subtenon space) and intraocularly (vitreous) and then processed for histological study. For that, the rats were divided into two groups: intravitreal injection (n= 21) and subtenon injection (n=33). In both cases, the right eye of each animal was injected under general anesthesia with blank PEA Ms and fluorescent PEA Ms suspended in PBS. The injections were done with a 30G without dead

space volumen needle fitted to a sterilized 10 µL (for intravitreal) or 25 µL (for subtenon) low dead space Hamilton syringe. For intravitreal injections 1, 2 or 3 µL of PEA Ms suspension were injected just behind the limbus to avoid damaging the lens. For subtenon injections 25 µL of the PEA Ms suspension were injected in the superotemporal quadrant. The intraocular pressure (IOP) of the rats was measured under deep anaesthesia in both eyes with a rebound tonometer (Tono-Lab, Tiolat, OY, Helsinki, Finland) prior and after PEA Ms injection. Animals were sacrificed at two time points: immediately and 24 hours after microsphere injection.

Before and after injection, clinical evaluation of the rat eyes were done under a surgical microscope (Leica M 500-N, Leica Microsystems, Schweiz AG) by two independent ophthalmologist in a masked procedure. At each examination the conjunctiva, cornea, anterior chamber, lens, vitreous and retina were examined. Clinical signs were recording according to a scoring system. At each examination the intraocular pressure was measured while rats were under deep anesthesia. At all time points, 3 to 4 consecutive readings were perforemed for each eye and were averaged.

Tissue processing

The rats were deeply anaesthetized, perfused transcardially through the ascending aorta first with saline and then with 4% paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.4). The animals were sacrificed with an intraperitoneal overdose of pentobarbital (Dolethal Vetoquinol®, Especialidades Veterinarias S.A., Alcobendas, Madrid, Spain). The orientation of each eye was carefully maintained with a suture placed on the superior pole immediately after deep anaesthesia and before perfusion fixation. Moreover, upon dissection of the eye, the insertion of the rectus muscle and the nasal caruncle were used as additional landmarks. The eyes were post-fixed for 2h in the same fixative and kept in sterile 0.1 M PBS. Subtenon injected eyes were processed for cryosections and eyes receiving intravitreal injection for both, cryosections and as retinal whole-mounts. For cryosections, the lenses were removed and the eye cups were cryoprotected by overnight incubation in 30% sucrose at 4ºC, after which they were embedded in Tissue-TeK O.C.T compound. For retinal-whole mount the cornea, iris and lens were removed and the retinas extracted from the resulting eye cup.

Localization of microspheres after subtenon and intravitreal injections

In order to analyze PEA Ms behavior and to ascertain their ocular location after injection, the retinal whole-mounts and some cryosections were observed unstained (mounted with PBS/glycerol) both, under light and fluorescence microcopy. In addition, some cryosections were stained with hematoxylin-eosin (HE), dehydrated and mounted with water-free mounting medium (DPX. Merck, Germany).

Ocular tissues were analysed and photographed with an imaging microscope (Axioplan 2; Zeiss, Göttingen, Germany) equipped with appropriate filters for fluorescence-emission spectra of 515/65 nm (Filter set 17, Zeiss), 647/70 nm (Filter set 64, Zeiss) and 445/45 nm (Filter set 49, Zeiss) and Nomarski Interference Contrast illumination technique.

RESULTS

Polymer characterization

The obtained PEA product was of number average molecular weight (Mn) of 49 kDa and narrow dispersity index ($Đ_M$) as revealed by GPC analysis (Table 1).

Table 1 Polymer characterization

	Mn (kDa)	Glass transition temperature	$Đ_M$
PEA	**49**	**49 ^{0}C**	**1.56**

The ^{1}H NMR spectrum was in full agreement with the anticipated chemical structure of the polymer. The ^{1}H NMR spectrum of the purified polymer is in agreement with the chemical structure as shown in Figure 3.

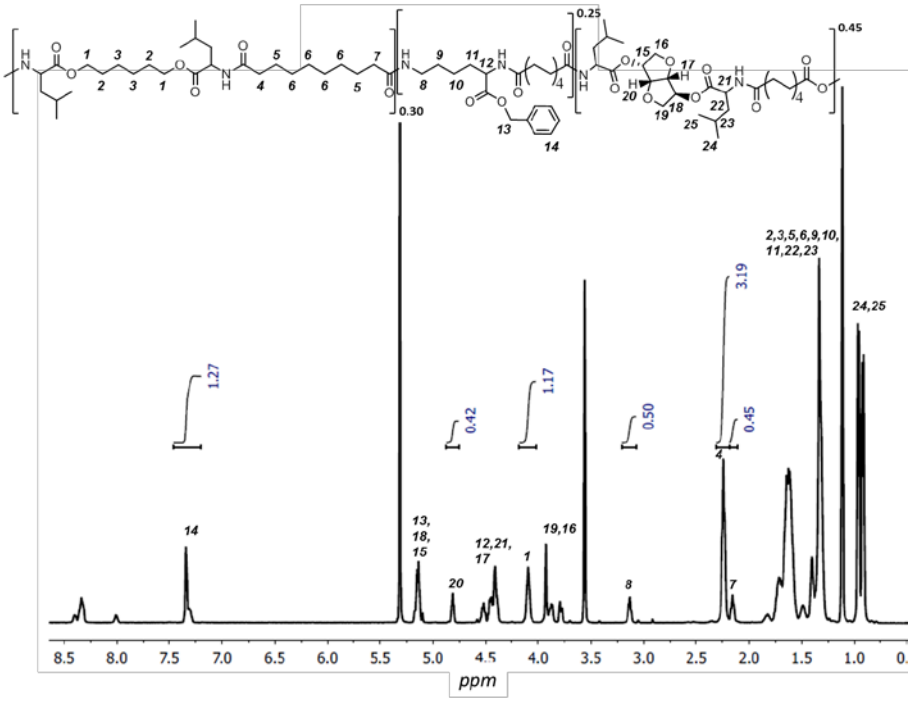


Figure 3 ^{1}H NMR spectrum recorded in deuterated ethanol, 500 MHz. The NMR data are in full agreement with the anticipated chemical structure of the polymer.

The aromatic signals 14 at 7.35-7.25 ppm correspond well to aliphatic multiplet at 3.20-3.10 ppm indicating no side ester groups have been lost in the polycondensation reaction. The signal of protons 4 shifted to lower field than protons 7. This allows differentiating between the amide linkages involving alpha and epsilon amino groups in the polymer chain. The characteristic chemical shifts for 1,6-hexanediol (m, 4.20-4,05 ppm), lysine (m, 3.20-3.10 ppm) and 1,4:3,6-Dianhydro-D-sorbitol (s, 4.80 ppm) allow for determination of the relative molar ratio of the amino acid containing building blocks (Fig 3). The results confirmed the very good agreement between actual and theoretical copolymer composition. The ^{1}H NMR spectrum further actual relative ratio between ester and amide bonds in the polymer chain to be calculated which is 1:1.33.

Microspheres characterization

The linearity of the method used to quantify dexamethasone was linear over the range 2-20 µg/mL (intercept -46395, slope 355329, coefficient of determination 0.999). Accuracy ranged between 98.4 and 101.3%, and intra- and interday precision determined by percent coefficient of variation, was 2.18% and 2.16%, respectively.

The microencapsulation procedure used led to a high yield in all cases (79.1 ± 6.15%). Microsphere' morphology examined by SEM revealed spherical particles with no pores and smooth surface (Fig 4). The mean particle size obtained ranged from 10-20 µm (15.0 ± 6.4 µm for blank PEA Ms and 14.2 ± 6.1 µm for DX-PEAIII Ms). The encapsulation efficiency of DX was 85.1 ± 0.9% (181.0 ± 2.4 µg DX/mg Ms). The number average molecular weight of PEA Ms was similar to the one found for the raw PEA material (Mn=48.3 ± 3.7 KDa for blank PEA Ms and Mn=49.2 ± 1.4 KDa for DX-PEAIII Ms).

Sterilization of microspheres

The morphology of microspheres was not modified by the sterilization procedure (Fig. 4) and no changes in the mean particle size were detected (15.2 ± 6.6 µm for blank PEA Ms and 15.0 ± 6.1 µm for DX-PEAIII Ms). Sterilization by γ-irradiation did not modify the drug content (183.2 ± 1.6 µg DX/mg Ms, p=0.86). Furthermore, solubility of the polymeric particles in THF did not change as well suggesting that the gamma irradiation at the sterilization conditions does not result in polymer branching or crosslinking. The GPC results revealed a minor change in the polymer molecular weight. The Mn of the polymer sample after sterilization was 14 % lower than the sample analyzed before gamma-irradiation suggesting limited chain cleavage.

In vitro release studies

The release rate of DX from sterilized and non-sterilized microspheres is shown in Fig 5. For non-sterilized microspheres, the release profile was characterized by an initial burst of 17.7 ± 0.6 µg DX/mg Ms (9.7 ± 0.3%) released in the first 24 h, followed by a short rapid release period of one week and a second long period of slow release up to 90 days. The cumulative DX release at day 90 was 53.9 ± 5.7% of the encapsulated drug (97.8 ± 11.1 µg DX/mg Ms). The release profile of sterilized and non-sterilized Ms showed similar results since the calculated similarity factor f was 98.3, which indicates that the average difference between the two release profiles was no more than 2%.

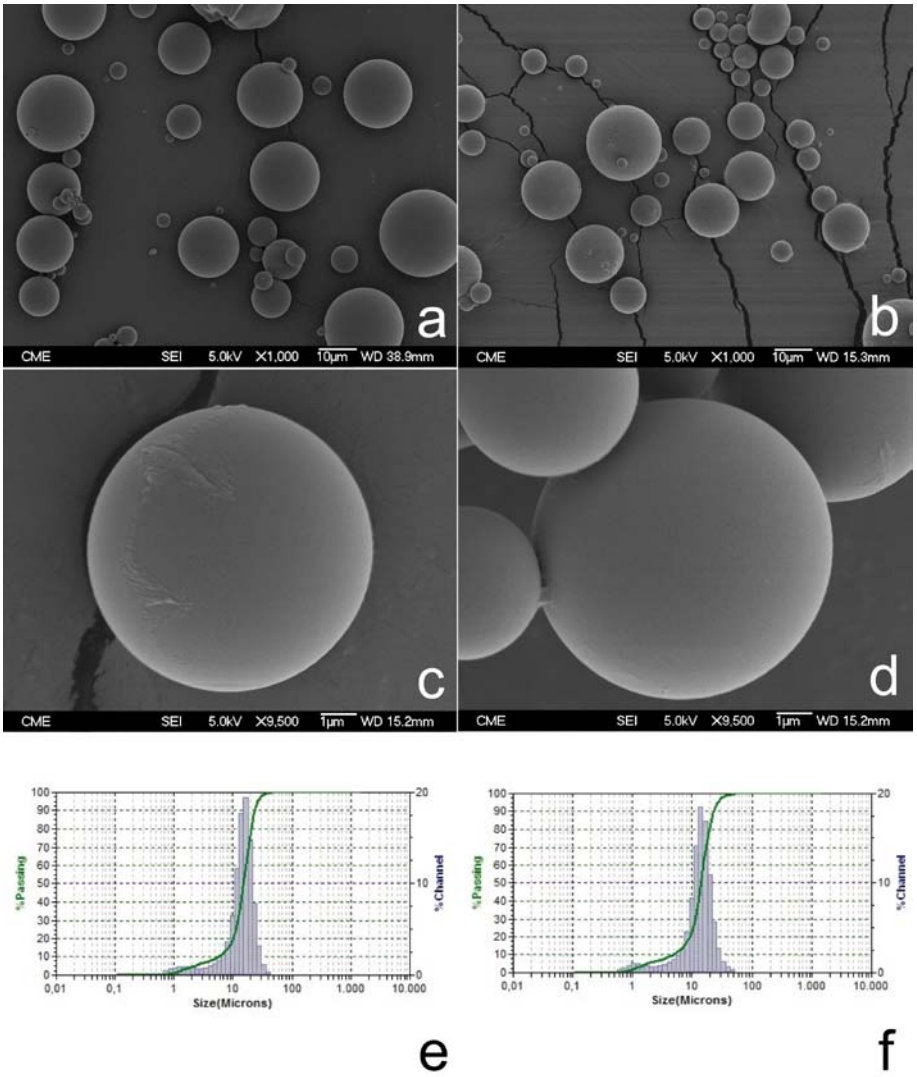
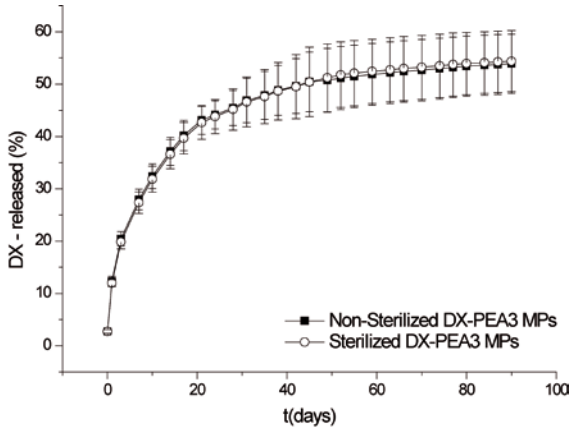


Figure 4 SEM images and particle size distribution of non-sterilized (a, c and e) and sterilized (b, d and f) DX-loaded PEA microspheres.



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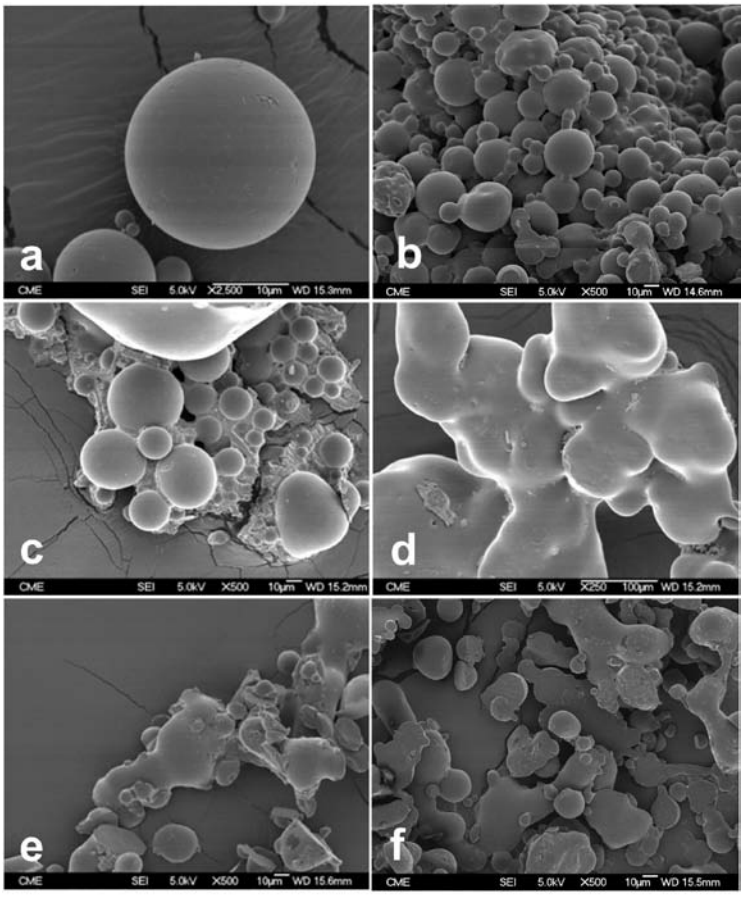
550

551 **Figure 5** Cumulative percent of dexamethasone released versus time of non-sterilized (-■-) and
552 sterilized (-o-) PEA microspheres. PBS was used as release media (pH 7.4, 37ºC).

553

554 Microspheres only kept their spherical morphology during the first day after incubation in PBS.
555 Then, a morphological change was produced and microspheres remodeled to an unshaped
556 depot (Fig. 6).

557



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560 **Figure 6** SEM images of PEA microspheres incubated in PBS, at different time points: (a)
561 before incubation, (b) t=1 hour, (c) 24 hours, (d) 3 days, (e) 1 week and (f) 2 weeks.

Kinetic modeling of dexamethasone release from microspheres

A pharmacokinetic model was built in order to predict dexamethasone concentration in rabbit vitreous during dexamethasone release from the PEA microspheres. Fig. 7 shows comparison of the experimental *in vitro* release data and simulated release based on the fitting of the release data and derivatization of the fitted curves. Finally, different dexamethasone loading doses were used in the in vivo dexamethasone model (Fig. 2), in order to simulate drug concentrations in the vitreous (Table 2, Fig. 8).

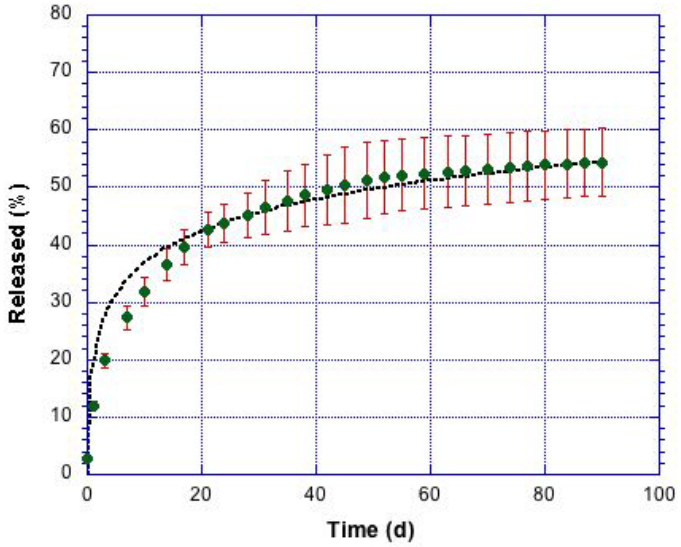

Figure 7 The *in vitro* release data (± S.D.) of dexamethasone from PEA microspheres versus the fitted dexamethasone release.

Table 2 The predicted dexamethasone concentrations (µg/ml) in rabbit vitreous at different doses delivered in the microspheres.

	Predicted Concentration (µg/ml)		
Dose	**C_{max}**	**42 days**	**90 days**
100 µg	2.25	0.012	0.0055
200 µg	4.42	0.024	0.011
400 µg	8.53	0.048	0.023
800 µg	15.88	0.096	0.044

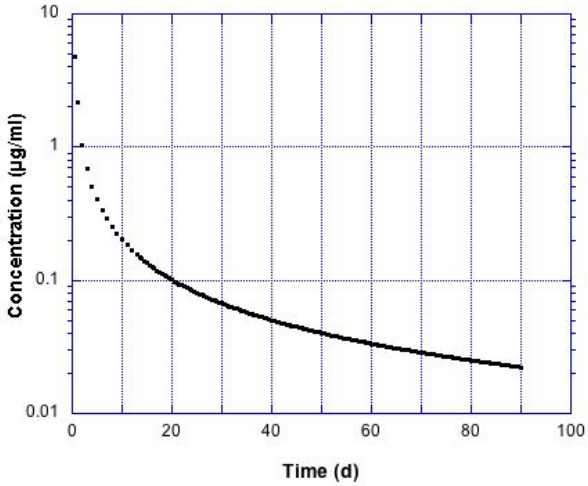

Fig. 8. Simulated time profile for free dexamethasone in the vitreous after administration in PEA miscrospheres.

Cytotoxicity

In vitro cytotoxic effects of PEA microspheres in RAW 264.7 macrophages are shown in Fig 8. Cell viability was not significantly affected in the presence of different concentrations of microspheres prepared with polyesteramide polymer (PEA) or PLGA whereas, treatment with PEI microspheres, used as positive control, caused a sharp drop in cell viability at 2 mg/mL.

Similarly, no signs of toxicity were seen in ARPE-19 cells after 5 hours of incubation, for both PEA and PLGA microspheres (Fig. 9) In contrast, poly-L-lysine showed high toxicity at all concentration (IC_{50} 17.3 ± 2.3 µg/mL).

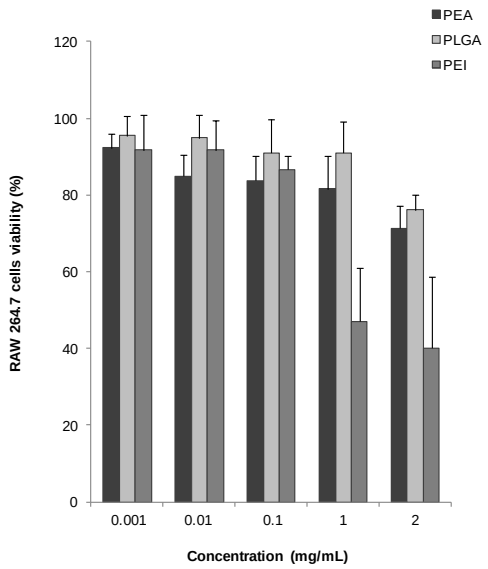

Figure 8 Effects of PEA on cell viability. RAW 264.7 cells were pre-incubated with PEA microspheres (0.001-2 mg/ml) for 20 h. Microspheres of polyethyleneimine (PEI) were used as positive control and PLGA was considered as a reference of non-toxic material. Cell viability was determined by the MTT assay. Values are expressed as mean ± coefficient of variation of three independent experiments.

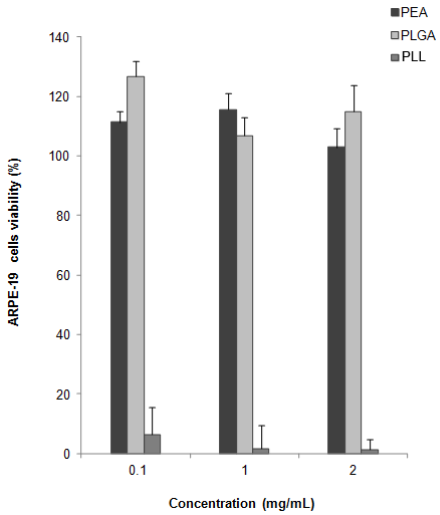

Figure 9 Percentage cell viability of ARPE-19 treated cell (± standard deviation). Cells were incubated with polyesteramide microspheres (PEA), poly(lactic-co-glycolic) acid microspheres (PLGA) or poly-L-lysine (PLL) for 5 h.

Handling of microspheres for in vivo injections

Under light microscopy PEA Ms suspended in PBS were easily visualized. Under fluorescence microscopy, unloaded PEA Ms had autofluorescence emission in the blue, green, and red spectral regions. However, in the red spectral region the emission intensity was lower than for dye-loaded microspheres (supplementary material).. PEA Ms: i) were inert and stable when contacted with distilled water (Fig. 10A), phosphate buffered saline (Fig. 10B), 4% paraformaldehyde (Fig. 10C,D) and 2% glutaraldehyde; ii) as expected PEA particles loose shape or dissolve in contact with Xylene (Fig. 10E), EtOH (Fig. 10F) or acetone ; iii) freezing at -20ºC (Fig. 10D) and heating at 60ºC does not impact particles re-dispersion, shape and polydispersity (supplementary material).

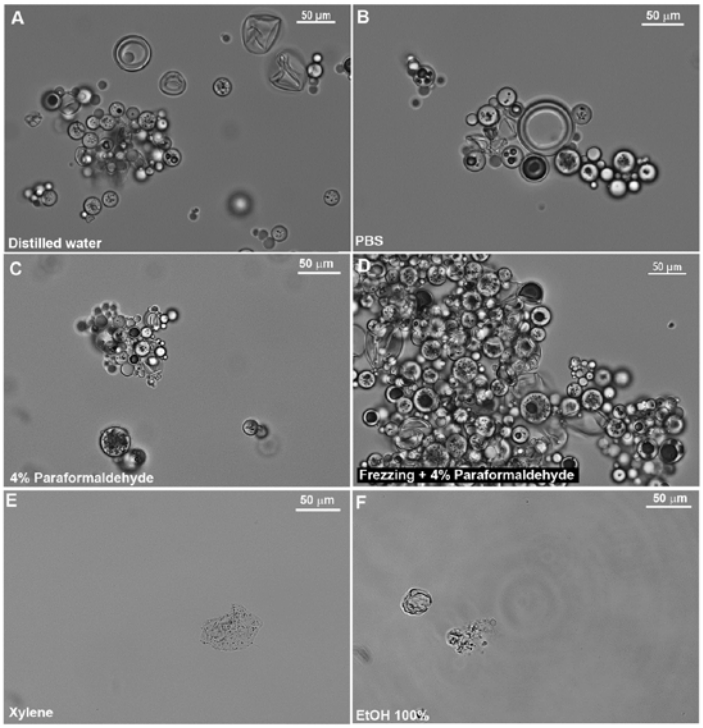

Figure 10 Effects on PEA microspheres of reagents, solutions and processes required to perform histological procedures. PEA microspheres were inert and stable when contacted with distilled water (2A), phosphate buffered saline (2B) and 4% paraformaldehyde (2C,D). In contrast, they were dissolved in contact with Xylene (2E) and EtOH (2F). They resisted freezing at -20ºC (2D).

Unloaded and dye-loaded PEA Ms had a tendency to aggregate both, at the eppendorf and inside the Hamilton syringe after suspension preparation, this feature being more pronounced in the dye-loaded Ms. This tendency increased with time, being greater 30 minutes after suspension preparation. Aggregation of dye-loaded PEA Ms precluded quantification in the Neubauer chamber cell counting (Fig. 11B). For freshly suspensions of unloaded PEA Ms, no significant differences in microspheres number released through different needle gauges (25G, 27G, 30G, 32G) were found (Table 3) (Fig. 11A). However, when the elapsed time between preparation and injection was longer, a tendency to aggregate was observed in Ms, being this feature more pronounced in dye-loaded Ms, whose counting was not possible with this method (Fig 11B). So, in order to assure a proper administration, the injection of Ms should be performed during the first 30 minutes after their dispersion.

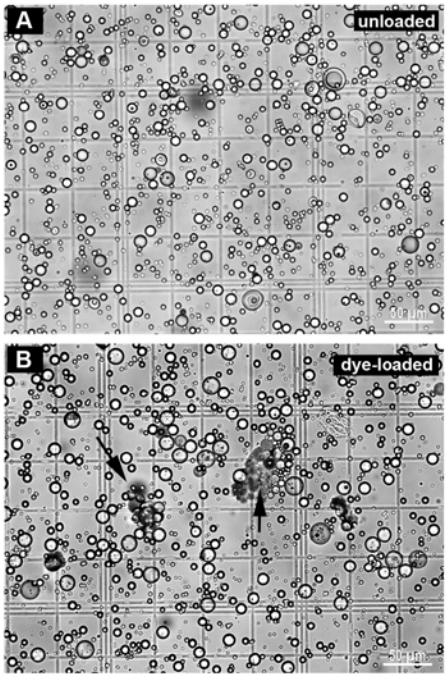

Figure 11 PEA microspheres quantification in the Neubauer chamber cell counting. The microphotographs correspond to Neubauer chamber loading through a 30G needle. Unloaded PEA microspheres (A) counting was possible. However, the tendency of dye-loaded PEA microspheres (B) to aggregate (arrow) precluded quantification with this methodology. Light microscopy.

Table 3 PEA microspheres quantification in a Neubauer chamber cell counting

Needle gauge	PEA microsphere number†
25G	304.48 ± 39.35
27G	322.80 ± 31.38
30G	333.56 ± 39.81
31G	316.88 ± 35.62

† mean ± standard deviation

Localization of microspheres after intravitreal and subtenon injections

No clinical signs of inflammation were observed in the anterior or posterior segment of the rats after subtenon or intravitreal injection. At the two time points of sacrifice it was observed that most PEA Ms aggregate and coalesce into depots, both, in the vitreous (Fig. 12G) and in the subtenon space (Fig. 12C,D,E,F,H), that contained a large amount of Ms. Intravitreal injection of 1-3µl of PEA Ms did not increased IOP.

In unstained whole-mount and cryosections (both mounted with PBS/glycerol), dye-loaded PEA Ms (Fig. 12A, B) and unloaded PEA Ms (Fig. 12E, H) preserved their morphological characteristics. However, during HE staining of the cryosections, alcohol dehydration dissolved the microspheres and a “ghost image” was observed, corresponding with the intraocular location of the microspheres (Fig. 12F). Under fluorescence microscopy, the autofluorescence of the ocular tissues hinder visualization of unloaded PEA Ms (Fig. 12H). Dye-loaded PEA Ms improved notoriously microspheres visualization (Fig. 12B,D).

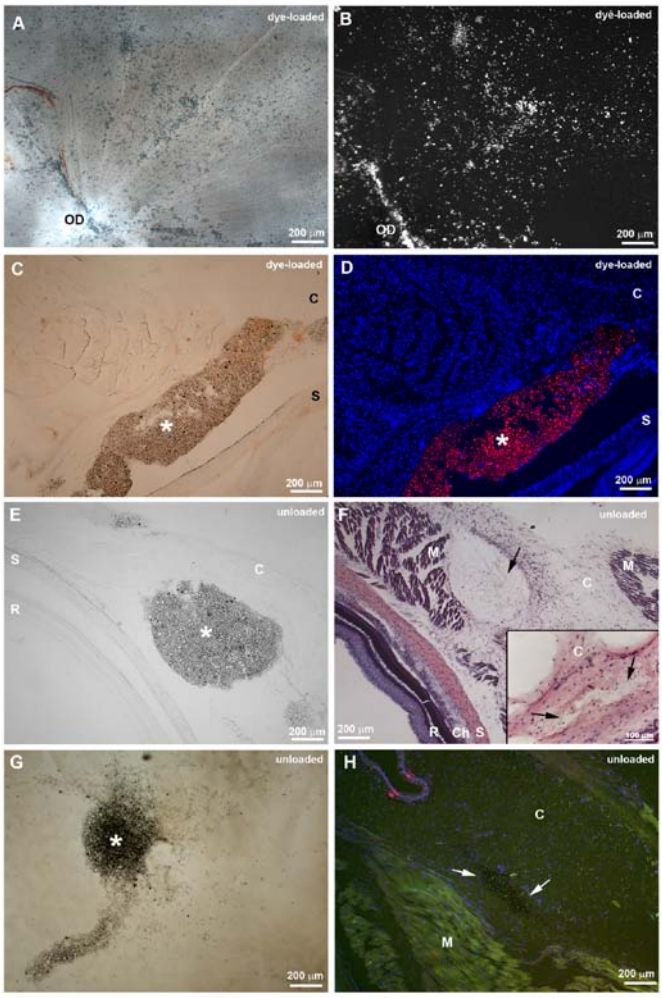

Figure 12 intraocular locations of dye-loaded and unloaded PEA microspheres immediately and 24 hours after injection. Whole-mounts (A,B,G). Cryosections (C,D,E,F,H). Light microscopy (A,C,E,F,G). Fluorescence microscopy (B, D, F). In unstained tissues mounted with an aqueous media (A-D, F-H), PEA microspheres preserved their morphological characteristics and coalesced into depots in the two locations tested: intravitreal (A, B, G) and subtenon (C, D, F, H). Tissue dehydration required to perform hematoxylin-eosine staining dissolved the microspheres but a “ghost image” corresponding with the intraocular site of the PEA microspheres (black arrows) remains (F). Although unloaded PEA microspheres were autoflorescent, the autofluorescence of the ocular tissues hinder unloaded PEA microspheres (white arrows) identification (H). In this regard, dye-loading (D) enhanced significantly

microsphere identification. OD: optic disc; C: conjunctiva; M muscle; R: retina; S: sclera; Ch: choroid.

DISCUSSION

Current developments in the therapy of ophthalmic chronic diseases are directed towards the use of new drug delivery systems that allow sustained therapeutic levels of therapeutic molecules at ocular target tissues. Due to the unique characteristics of ocular barriers, controlled drug delivery has become a challenge for scientists, especially when the target site is located in the posterior segment of the eye. Among the intraocular drug delivery systems, implants and microspheres have demonstrated to provide long term delivery of the active substance after their administration and the utility of microspheres as drug delivery systems for intraocular administration has been previously reported [18-20, 30]. One of their main advantages is that they can be administered as a conventional injection using a small gauge needle. Furthermore, depending on the patients' needs, a properly dose of the active substance can be adjusted by administering a determined amount of microspheres, allowing a personalized therapy. In this experimental work, the use of polyesteramide polymers (PEA), a new generation of biodegradable materials, to prepare microspheres as drug delivery systems for the back of the eye, has been evaluated.

PEA microspheres (Ms) were prepared with a technique based on the emulsion- solvent evaporation method. The mean particle size obtained ranged from 10 to 20 µm, suitable to be administered without surgery by conventional injection as suspension through standard needles (27-34G) [18, 19]. One of the critical aspects of drug delivery systems for the back of the eye is the tolerance. In general, cells can endocytose particles that are below 200 nm in diameter. However, macrophages and retinal pigment epithelial (RPE) cells are phagocytic cells that may take up even particles in the micrometer size range [21]. Thus, it is important to study the toxicity in these cell types that will be exposed to the particles upon their internalization. In the present work, the ARPE-19 cell line served as the model for RPE cells [22]. *In vitro* cytotoxicity of PEA microspheres was tested by the MTT technique with microspheres dispersed in HBSS. In a culture of macrophages (RAW 264.7), after 20 hours of exposure time, PEA Ms resulted to be well tolerated, giving viability values higher or equal to 80% at all the concentrations that were tested (0.001-2 mg/mL). Similarly, the PEA microspheres (0.1-2 mg/mL) did not show any signs of toxicity in ARPE-19 cells after 5 hours of incubation.

PEA microspheres were sterilized by γ-irradiation at a dose of 25KGy [4]. Sterilization was performed at low temperature to reduce the effects on the polymer, since it is known that γ-irradiation can affect the properties of bioresorbable polyesters [15]. No significant changes on the morphology of microspheres, loading and release of dexamethasone were observed due to sterilization under the conditions employed in this work. The polymer analyses confirmed that

particles sterilization under the experimental conditions results in only minor chain scission of polymer chain and do not affect important material characteristics such as morphology and solubility

Pharmacokinetic simulations were conducted in order to predict the intraocular concentrations of dexamethasone during drug delivery from the PEA microspheres. *In vitro* release data from PEA microspheres and pharmacokinetic information from rabbit vitreous were utilized in the model. Taking into account the encapsulation efficiency of the microencapsulation procedure (181 µg dexamethasone/mg PEA microspheres), the intravitreal injection volume in rabbit (100 µL) and the microsphere concentration of 25 mg/mL, the highest possible dexamethasone dose in the microsphere injection would be around 450 µg. In the simulations, 400 µg of dexamethasone resulted in C_{max} of 8.5 µg/mL and the simulated concentration at 3 months was 23 ng/ml. We can compare these values after Ozurdex implant delivery (700 µg dexamethasone) in cynomolgus monkeys: C_{max} was 213 ± 49 ng/mL (at 60 days) and at 6 months concentration was 1.3 ± 1.9 ng/mL [23]. In clinical studies Ozurdex was tolerated well and caused improvement in visual acuity for 180 days in patients with macular edema [24-26]. PEA microspheres seem maintain therapeutic dexamethasone concentrations in the vitreous for at least 3 months. Importantly, 400 µg dose of dexamethasone sodium phosphate as intravitreal solution injection in the rabbits did not cause any abnormalities during 2 months [17,27]. This suggest that the initial rapid release from dexamethasone microspheres is not expected to lead to dexamethasone borne toxicity. Intravitreal dexamethasone dose of 450 µg would require 2.2 mg of PEA microspheres that would result in the maximal PEA concentration of 1.47 mg/mL in the vitreous. This concentration was safe in the cellular MTT assays, but further *in vivo* studies are needed to prove the duration of activity and safety of PEA microspheres with dexamethasone.

In this paper PEA Ms compatibility with the steps required for intraocular and periocular injection (known routes to deliver drugs to the posterior segment of the eye) [28] has been studied in detail. Furthermore, the polymer interaction with histological analysis reagents has been investigated as first step of comprehensive biocompatibility assessment. Several reagents are required for presevering (fixative solutions) and sectioning the tissues (embedding techniques) during histological processing. PEA Ms were compatible with the two main fixatives used in histology, paraformaldehyde (for light microscopy) and glutaraldehyde (for electron microscopy).

PEA Ms loose shape or dissolved during the dehydration steps required for paraffin and epoxy resin embedding techniques. Therefore, cryosections seems to be the most suitable method for PEA Ms visualization under light microscopy. Although PEA Ms can be easily recognized in unstained sections mounted with an aqueous media, biocompatibility assays require tissue staining. The present study reveals that staining techniques including dehydration in their protocols (i.e., HE used in the present study) affect PEA Ms. Thus, in order to analyze PEA Ms

biocompatibility, consecutive unstained (mounted with PBS/glycerol) and stained cryosections must be simultaneously analyzed; the former for microsphere location and the latter for tissue tolerability analysis.

PEA Ms can be released into ocular tissues through different gauge needles (25G, 27G, 30G and 32G). Given that no significant differences in the number of PEA Ms were found between the different needles gauges analyzed, a 30G was selected for intraocular injection as it is the most frequently used in human clinical settings. Due to the tendency of microspheres to aggregate (Fig. 3A), injection during the first 30 minutes following suspension preparation assured a properly administration. The most appropriate volume of PEA Ms for intravitreal injection in rats was 3 µl, since this volume achieves enough amounts of microspheres in the vitreous humor without increased IOP. Twenty four hours after intraocular injection both dye-loaded PEA Ms and unloaded PEA Ms retain their morphology and coalesce into depots in the vitreous and in the subtenon space. Such depots contained a large amount of Ms suggesting that the PEA Ms are able to reach properly the injection site. Chromoionophore II-containing PEA Ms were not suitable for in vivo assays given that due to their great tendency to aggregate, it is not possible to ascertain that a sufficient and reproducible number of Ms reach the injection space.

CONCLUSIONS

Amino acid based polyesteramides were successfully formulated into microspheres (~15 µm), which showed good *in vitro* tolerance with macrophages and retinal pigment epithelial cells. The microspheres can be readily sterilized by gamma irradiation and administered using 27-32G needles within 30 min of suspension preparation. Loading and release studies with dexamethasone revealed high drug encapsulation efficiency (~85%) with a controlled delivery for 90 days. Pharmacokinetic simulations indicate that these microspheres would provide a release of the drug in rabbit eyes up to 3 months. After 24 hours, the injected PEA Ms retain their morphology and coalesce into depots in the vitreous and in the subtenon space. The large amount of Ms located into the depots suggested that PEA Ms were able to reach properly the injection site. It can be concluded that these polyesteramide microspheres provide an alternative delivery system for controlled delivery of drugs to the eye.

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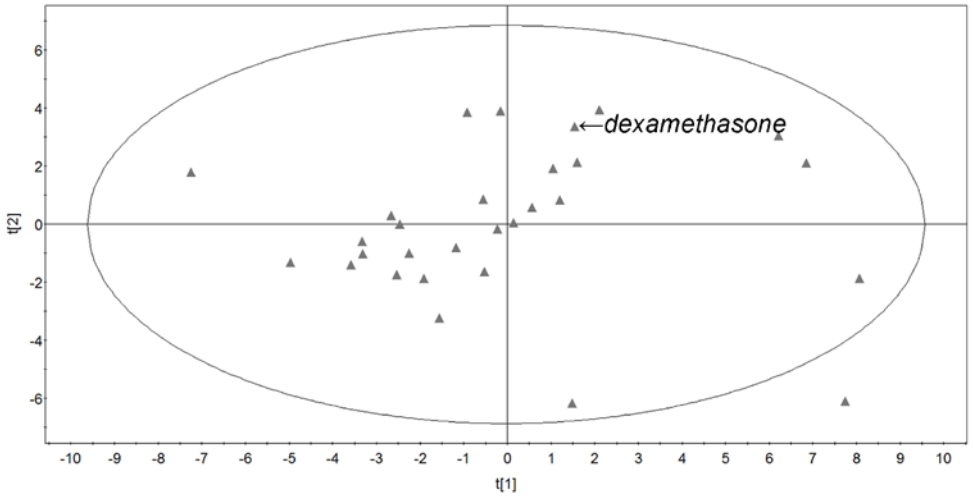
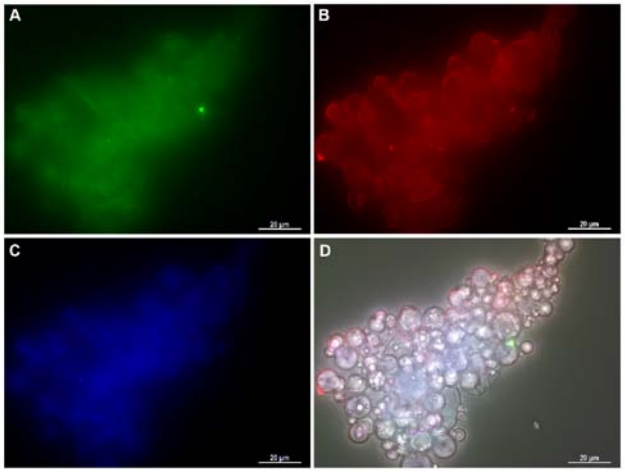
Supplementary material



Supplemental figure The PCA score plot of the training set of the intravitreal clearance model {{del Amo et al. 2015, http://dx.doi.org/10.1016/j.ejpb.2015.01.003 }}. The full triangles show the chemical space of the compounds that were used to train the model. Dexamethasone is inside the chemical space ellipse indicating that the model is applicable for dexamethasone.



Supplemental figure Unloaded PEA microspheres. Morphological appearance under fluorescence (A-C) and light (D) microscopy. PEA microspheres had autofluorescence emission in the green (A), red (B) and blue (C) spectral regions. Nomarski interference contrast (D).

Supplemental table PEA microspheres preservation after 5 minutes of contact with reagents and solutions used in common tissue staining methods. Light microscopy. Regents and solutions assayed did not affect the autoflourescence properties of the unloaded PEA microspheres.

Reagents and solutions*	**PEA microsphere preservation****
Distilled wáter	+++
Phosphate buffered saline	+++
EtOH 100%	-
Acetone	-
Xylene	-
4% PF	++++
2% Glutaraldehyde 2%	++++
4% PF (5 min) + EtOH 100%	±
4% PF (5 min) + Xylene	±
4% PF (5 min) + EtOH (5 min) + Xylene	±
2% Glutaraldehyde (5 min) + Acetone	±

*100 µl of the reagent were added to the microsphere powder. **Subjective grading scale ranging from microsphere shape unaffected (++++) to microsphere dissolved (-). [PBS: Phosphate buffered saline; PF: paraformaldehyde

Supplemental table scoring system used to evaluate the rat eyes

Conjunctival Hyperemia	
No reaction, normal vessels,	0
Definitely injected vessels /Mild hyperemia	1
Diffuse crimson red / Intense	2
Diffuse beefy red	3
Conjunctival Swelling	0
No reaction	1
Mild edema	2
Intense edema	3
Cornea	
Normal cornea	0
Scattered o diffuse areas of opacity (Details of iris clearly visible)	1
Details of iris slightly obscured	2

No details of iris visible, size of pupil barely discernible	3
Opaque cornea: iris no discernible through the opacity	4
Anterior chamber haze	
Details of iris clearly visible	0
Details of iris slightly obscured	1
No details of iris visible, size of pupil barely discernible	2
Opaque cornea: iris no discernible through the opacity	3
Lens	
Clear	0
Cataract	1
Vitreous	
Absence of inflammation	0
Isolated cells that allow to visualize the main vessel	1
Mild to moderate haze with most of the retina obscured	2
Severe haze and infiltration of the retina totally obscured	3
Retinal detachment	
Absence	0
Presence	1
Retinal and vitreous hemorrhages	
Absence	0
Presence	1

*Modified from Einmahl, 2001; Rincon, 2006 and Diebold, 2007

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