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Poly(ortho ester) Nanoparticle-Based Targeted Intraocular Therapy for Controlled Release of Hydrophilic Molecules

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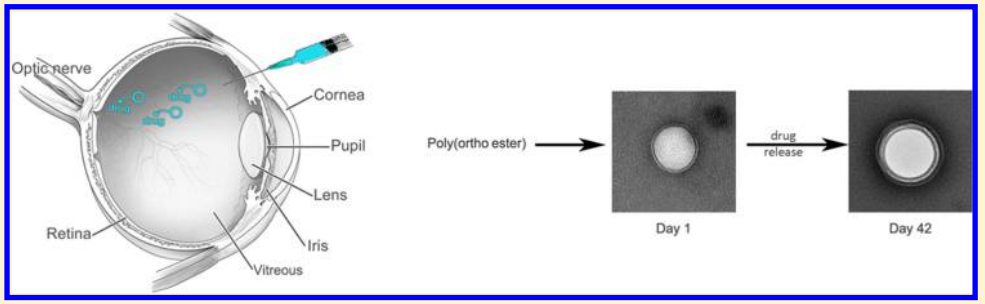

ABSTRACT: Development of an efficient intraocular drug delivery nanosystem remains the most difficult challenge to attain a prolonged therapeutic effect at the site of drug action. The purpose of this work was to develop a biodegradable, long-term sustained release, and biocompatible nanoparticulate system to treat various intraocular diseases. To attain the objectives, poly(ortho ester) (POE), a hydrophobic, surface erodible, and nontoxic polymer, was selected for fabrication of nanoparticles for the first time using a double emulsion solvent evaporation method. The influence of POE molecular weight on particle size, polydispersity index, zeta potential, drug content, in vitro release, degradation, in vitro cytotoxicity, and cell uptake studies was investigated. Drug-loaded nanoparticles had a spherical shape with an average particle diameter from 241 to 298 nm and zeta potential values from −8 to −11 mV. Encapsulation efficiency ranged between 21 and 63%, depending on the type of the water-soluble molecule used. Approximately 20−30% of the loaded drug was released over a period of 14 weeks. The drug release and degradation profiles of nanoparticles followed perfect zero-order kinetics confirming the POE-surface erosion mechanism. In vitro cytotoxicity and cell uptake studies revealed the cyto-compatible nature and nonendocytic behavior of POE nanoparticles. Collectively, POE nanoparticles are a very promising vehicle for sustained delivery of therapeutics to the back of the eye.

KEYWORDS: *biodegradable nanoparticles, poly(ortho ester), water-soluble molecules, zero-order, cell-uptake*

1. INTRODUCTION

There are several modes by which drugs can be administered to the posterior segment of the eye including via systemic circulation, eye drops, transscleral delivery, or intraocular injections. Systemic administration suffers from lack of targeting and subsequent off-target effects manifesting in clinical adverse events and/or side effects. Both eye drop administration and transcleral delivery are noninvasive; however, the many physical barriers that are present in the eye limit the efficiency of drug delivery to the posterior tissues of the eye.[1−4]

Several pathologies of the eye still require new methods of drug administration to enhance the efficacy of treatment. Compliance is also problematic, particularly among patients who have chronic diseases such as glaucoma and refractory chorioretinal diseases, including uveitis, macular edema, neovascular (wet) and atrophic (dry) age-related macular degeneration (AMD), and retinitis pigmentosa.[5] For example, adjunctive treatment of glaucoma filtration surgery with anti-inflammatory and antiproliferative drugs, such as 5-flourouracil and mitomycin C, are administered topically.[6] Moreover, it has been reported that nearly 50% of glaucoma patients discontinue all topical ocular hypotensive therapy within six months,[5] thus contributing to very poor compliance of daily topical ocular medications. Comparatively, for the treatment of neovascular AMD and macular edema secondary to retinal vein occlusion, standard therapy includes monthly intravitreal injections of ranibizumab (Lucentis), an antivascular endothelial growth factor monoclonal antibody fragment.[7] In addition to the expense, frequent intravitreal injections might cause complications, such as endophthalmitis and retinal detachment.[8] Therefore, suitable drug delivery systems for increasing patient and doctor's convenience are urgently needed.

Although new potential therapies for the treatment of the diseases of the retina and choroid are being discovered, due to the invasiveness of intraocular injections and the potential complications that may ensue repeated dosing, a nontoxic

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biodegradable sustained drug delivery system for treatment of the intraocular diseases is highly desirable.[1,2] A sustained delivery system would both limit the number of repeated injections necessary to preserve vision, as well as circumvent high fluctuations in drug concentrations that are experienced following injections of the drug alone. Such emerging methods for sustained drug delivery to the posterior segment include biodegradable polymeric-controlled release injections and implants, nanoparticulates, microencapsulated cells, and iontophoresis.[3,9−16]

Many biodegradable synthetic polymers such as polylactic acid, poly lactic and glycolic acid copolymer, polycaprolactones, polyanhydrides, and poly(ortho ester) (POE) have been used to develop drug delivery carriers for intraocular applications.[13−15,17−19] POE polymer is a hydrophobic polymer, degraded by surface erosion confined to the polymer−water interfaces, and follows zero-order drug release kinetics, rather than drug diffusion, when placed in a biological environment. Even though the ortho ester linkages of POE are very reactive, the polymer is highly hydrophobic. Therefore, the amount of water that can penetrate through the polymer is very limited, and thus, the erosion rate is extremely slow. Clearly, the synthesis of drug delivery systems that have realistic delivery times requires the development of a means of accelerating the erosion rate in a controllable and reproducible manner. To accomplish this, one can incorporate a latent acid such as lactic acid or glycolic acid into the polymer resulting in a fourth generation POE polymer (POE IV).[20] Importantly, the degradation products of POE caused by surface erosion are nontoxic for the intraocular applications.[17]

In our study, we use lactic acid to facilitate the erosion process, which causes sustained drug release following zero-order kinetics. The degradation rate of POE depends on the molar ratio of latent acid in the polymer chain, hydrophilicity/lipophilicity of the diol, and molecular weight of the polymer.[21−23] By adjusting the molar ratio of latent acid, and a suitable diol, the degradation time of POE and subsequently its release profile can be varied accordingly.[24−26] Schwach−Abdellaoui et al. demonstrated that the rate of erosion is directly proportional to the molar concentration of lactic acid dimer incorporated into the polymer backbone.[21] In the present study, we carefully selected a flexible, hydrophobic 1, 10-decanediol and a minimum concentration of lactic acid (5 mol %) as a hydrophobic latent group to generate a slowly degradable POE polymer to prolong the drug delivery. The present study reports, for the very first time, the preparation of POE nanoparticles, characterization of their physical properties based on the molecular weight of polymer, and also their ability to encapsulate various kinds of water-soluble molecules such as epinephrine, rhodamine 6G, and bovine serum albumin (BSA). It also describes the influence of polymer molecular weight on in vitro release behavior of a small drug molecule, epinephrine. To assess the utility of POE nanoparticles as an effective drug delivery carrier for treatment of intraocular diseases, cytocompatibility and nanoparticle cell uptake studies were also evaluated.

2. MATERIALS AND METHODS

2.1. Materials. Poly(vinylalcohol) (PVA), 1,10 decanediol, D,L-lactone, poloxamer 188, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rhodamine 6G, (−)-epinephrine (+)-bitartrate salt, acetone, dichloro methane, tetrahydrofuran, and dimethyl sulfoxide (DMSO) were obtained from Sigma Aldrich (St. Louis, MO). 3,9-Divinyl-2,4,8,10-tetraoxaspiro[5.5]undecane was obtained from AK Scientific Inc., USA. Bovine serum albumin (BSA) was purchased from Electron Microscopy Sciences (Hatfield, PA). Lactate assay kit was purchased from BioVision (Milpitas, CA). Dulbecco's Modified Eagle Medium was purchased from Fisher Scientific (Fair Lawn, NJ). Oregon Green 488 DHPE and To-Pro-3 iodide were purchased from Invitrogen (Grand Island, NY). Human Embryonic Kidney cell lines (HEK-293) and Eagle's minimal essential medium (EMEM) were obtained from ATCC (Manassas, VA). rMC-1 Müller cells (Müller cells) were generously provided by Dr. Vijay Sarthy (Northwestern University).

2.2. POE Nanoparticle Preparation. Poly(orthoester) polymer was synthesized and characterized as described in the literature.[27] Briefly, 1,10-decanediol-lactate (2.5×10 mol), 1,10-decanediol (47.5×10^{-3} mol) were dissolved in anhydrous tetrahydrofuran. Subsequently, 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5.5]undecane (DETOSU) (50×10^{-3} mol) was added under argon atmosphere and the polymerization initiated by the addition of six drops of p-TSA w/w I % solution in THF. The exothermic reaction produced polymer almost spontaneously. The stirring was maintained for 1 h at room temperature, and the polymer then precipitated with methanol containing five drops of TEA to stabilize the polymer. After isolation, the polymer was dried at 40 °C under vacuum for 48 h.

Blank POE nanoparticles were prepared using a water-in-oil−water (W/O/W) double emulsion solvent evaporation technique with minor modifications.[28] Briefly, POE (90 mg) and poloxamer 188 (1.5 mg) were dissolved in 3 mL of 8:2 dichloromethane and acetone mixture. The organic mixture was added to 300 μL of an aqueous solution containing 2.5% w/v PVA and sonicated over an ice bath using a micro tip probe sonicator (S-4000; Misonic, Inc., Newtown, CT) at 65% intensity for 90 s to form a W/O primary emulsion. This primary emulsion was added dropwise under sonication to 18 mL of aqueous solution containing 2.5% w/v PVA to form a W/O/W secondary emulsion. The organic solvents were allowed to evaporate overnight by stirring over a magnetic stir plate. Nanoparticles thus formed were collected by ultracentrifugation (WX Ultra 80, Thermo-Scientific Sorvall Ultracentrifuge, Asheville, NC, USA) at 20 000 rpm, 20 min, 25 °C, and then washed three times with distilled water to remove unincorporated drug/dye and emulsifiers. The final product was dried by lyophilization at 0.002 mbar, −50 °C for 48 h (Freezone, Labconco Corporation, Kansas, MO). Drug/dye-loaded nanoparticles were also prepared in the same method mentioned above, where drug (20 mg)/dye (2 mg) was dissolved in inner aqueous solution.

2.3. Physico-Chemical Characterization of POE Nanoparticles. Particles were characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM). DLS measurements were conducted with particle suspensions (1 mg/mL) in Millipore water with a Malvern Nano-ZS (Malvern Instruments, Worcestershire, U.K.). The morphology of POE nanoparticles was documented using a transmission electron microscope (JEM-2000 EX II Electron Microscope, JEOL, LTD, Tokyo, Japan) using an acceleration voltage of 60 kV. Two microliters of POE nanoparticles (0.1 mg/mL) was placed at the center of a copper grid and dried in a desiccator for 24 h. Grids were visualized under the electron microscope at a magnification of 100 000×.

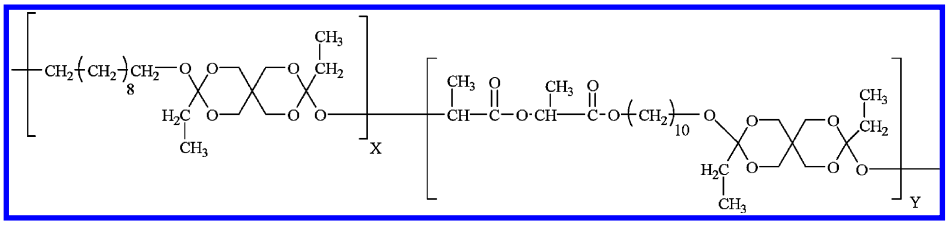

Figure 1. Chemical structure of POE (*X* is 95 mol % and *Y* is 5 mol %).

2.4. Determination of Encapsulation Efficiency. The encapsulation efficiencies of epinephrine, rhodamine 6G, and BSA in the drug-loaded nanoparticles were determined by dissolving 10 mg of drug-loaded nanoparticle powder in 2 mL of DMSO/deionized water (DIW) (1:1). Samples were rotated for at least 24 h at 50 rpm to ensure complete dissolution in aqueous DMSO solution. Empty nanoparticles were treated identically. The concentration of epinephrine and BSA in the resulting solution was determined by measuring the absorbance at 230 and 280 nm, respectively, in a spectrophotometer (MQX 200, Bio-Tec Instruments, Winooski, VT, USA). The rhodamine 6G concentration was measured at excitation/emission =528/560 nm using micro plate reader (FLx800, BioTek Instruments, Winooski, VT, USA). The obtained values were then subtracted from the absorbance values of empty POE nanoparticles. All samples were analyzed in triplicate.

2.5. In Vitro Epinephrine Release Studies. A dialysis technique was adapted to carry out the release experiments using a 5 kDa cut off membrane. Epinephrine release profiles were determined by suspending 30 mg of drug-loaded nanoparticles in 500 μL of PBS (pH 7.4). Samples were incubated at 37 °C while shaking at 50 rpm in an incubator shaker (C 24, New Brunswick Scientific, NJ). Every day, the release medium was removed and replaced with fresh buffer. The concentration of epinephrine in the supernatant was determined using the UV detection method described above. The absorption of supernatant collected from the empty POE nanoparticles was negligible at 230 nm throughout the release study. The linear range of the calibration curve was between 2−60 μg/mL, and the correlation coefficient of the standard curve was 0.9997 ± 0.00026. The amount of drug in each sample was summed with the amount from all previous time points to obtain the cumulative drug release amount. The total was then divided by the actual amount of drug in the nanoparticles to calculate the cumulative drug release percentage. Each release experiment was performed in triplicate.

2.6. Lactate Assay. Lactic acid release from the POE is a sign of the initialization of polymer degradation. Because of this, quantification of lactic acid released from POE nanoparticles is very essential to determine their degradation. Empty nanoparticles were dialyzed in the same way mentioned in section 2.5, and every day, the release medium was removed and replaced with fresh buffer. The amount of lactic acid release was quantified by using a lactate assay kit (BioVision Inc., Milpitas, CA) according to manufacturer's specifications.

2.7. In Vitro Cytotoxicity Measurement. In vitro cytotoxicity of nanoparticles was evaluated using an MTT assay[29] with minor modifications. Briefly, HEK-293 cells were seeded (4400 cells/well), and 100 μL of diluted nanoparticle (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 1 mg/mL) emulsions and positive controls, 1% Triton-X 100, and 80 μm H_2O_2 were added to each well and incubated with the cells for 48 h before being replaced by 160 μL of EMEM. Forty microliters of MTT reagent (5 mg/mL in PBS) was added to each well. After 2 h of incubation at 37 °C, 200 μL of DMSO was added to each well to dissolve the formazan crystals. After 5 min, the optical absorbance was measured at 570 nm and converted to percent viability relative to control (untreated) cells. All samples were tested in quadruplicate.

2.8. POE Nanoparticle Cell Uptake Study. *2.8.1. Qualitative.* In order to observe potential interaction of POE nanoparticles with cells, both Müller and HEK-293 cells were selected to evaluate the uptake of nanoparticles. Müller cells were selected due to their location within the retina of the eye and their potential influence when using POE nanoparticles to deliver an intraocular therapy. HEK-293 cells were commonly used to investigate nanoparticle uptake profiles. Cells were seeded in 24 well plates (BD Biosciences, Franklin Lakes, NJ) 48 h before assays were initiated. Rhodamine 6G-loaded nanoparticles were diluted in culture medium to 0.2 and 1 mg/mL and added to monolayers of HEK-293 and Müller cells. The cells were then incubated at 37 °C for 2 and 24 h. Cells were washed only once with PBS pH 7.4 and fixed in 1 mL of 1% paraformaldehyde solution (W/V) for 1 min, prior to labeling their nuclei with To-Pro-3 iodide (1:2000) in PBS for 10 min. After washing cells with PBS, cell membranes were labeled with 10 μg/mL of Oregon Green 488 DHPE for 10 min and washed thoroughly three times with PBS. Coverslips were mounted on slides, and images were taken on a confocal microscope (C1 Plus; Nikon, Tokyo, Japan).

2.8.2. Quantitative. Müller and HEK-293 cells were incubated with 0.2 and 1 mg/mL rhodamine 6G-loaded nanoparticles (20 μg of dye in 1 mg/mL nanoparticle emulsion) for 2 and 24 h as described for use in confocal microscopy. The cells were washed once with 1 mL of fresh medium, then with PBS twice. Cells were permeabilized by freeze−thaw cycles. Both permeabilized cells and washings were lyophilized at 0.002 mbar and −50 °C for 24 h (Freezone, Labconco Corporation, Kansas, MO), and obtained powders were suspended in a 2 mL, 1:1 mixture of DMSO and DIW. All samples were centrifuged at 20 000 rpm for 20 min. The clear supernatants were analyzed for rhodamine 6G at excitation/emission = 528/560 nm using a microplate reader (FLx800, BioTek Instruments, Winooski, VT). The linearity range of the calibration curve was between 2−10 μg/mL, and the correlation coefficient of the calibration curve was 0.9996 ± 0.0002. All samples were analyzed in triplicate.

3. RESULTS AND DISCUSSION

Biodegradable, biocompatible, and highly hydrophobic POE (Figure 1) was synthesized by an acid-catalyzed condensation of a diketene acetal (DETOSU) with 1,10-decanediol and 1,10-decanediol dilactate used as a latent acid.[27] Two different molecular weight POE polymers, 5 kDa and 22 kDa, were synthesized and utilized for the preparation and characterization of nanoparticles. Novel POE nanoparticles were

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Table 1. Physical Properties of Empty and Loaded POE Nanoparticles

	empty nanoparticles				water-soluble molecule-loaded nanoparticles						
POE mol wt (kDa)[a]	size[b] (nm)	PDI	ZP[b] (mV)	yield %	drug/dye	size[b] (nm)	PDI	ZP[b] (mV)	yield %	DL %	EE %
5	298	0.25	−8.12	70.5	Epi	241	0.112	−11.5	67.5	21.1	31.7
22	293	0.23	−8.28	71.6	Epi	242	0.104	−11.9	66.8	14.3	21.4
22					BSA	290	0.213	−22.7	73.9	44.2	63.3
22					Rho	263	0.189	−15.8	71.9	2	30

[a]mol wt = molecular weight; PDI = polydispersity index; ZP = zeta potential; DL = drug loading; EE = entrapment efficiency; Epi = epinephrine; Rho = rhodamine 6G. [b]One mg/mL nanoparticle emulsion was used to measure the size and zeta potential.

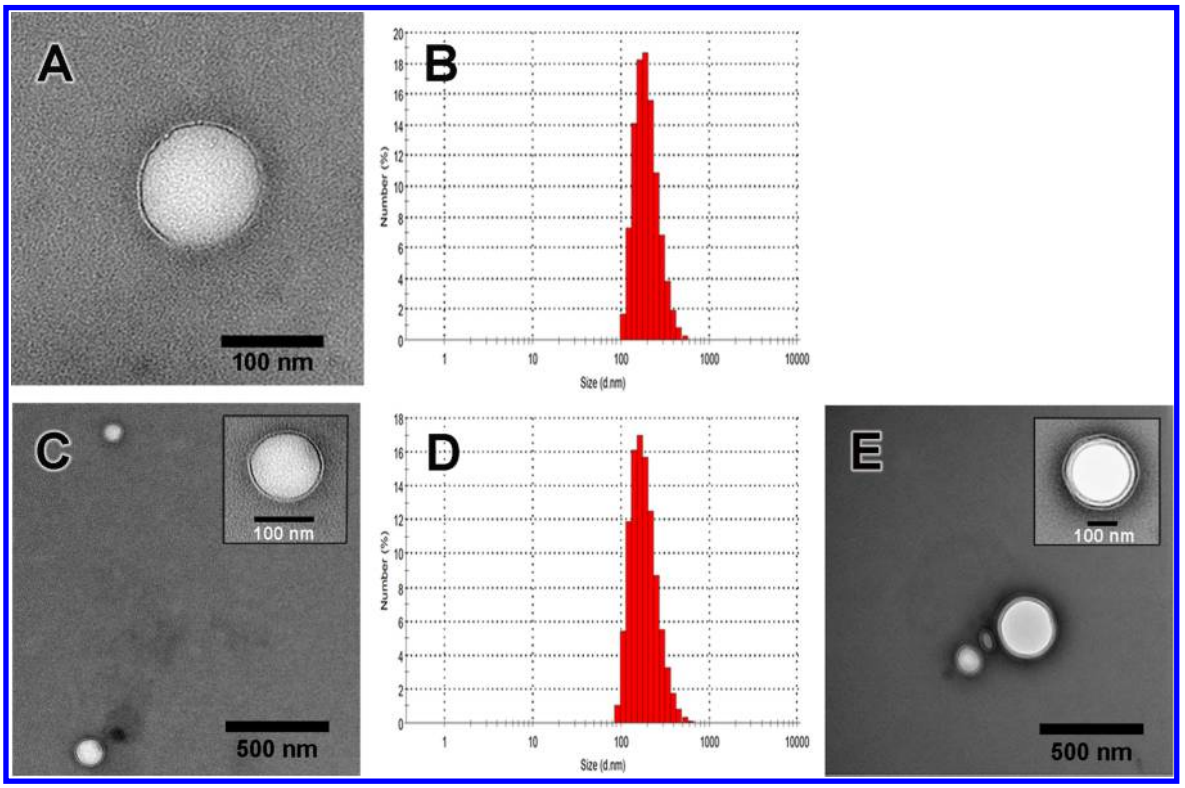

Figure 2. TEM images of empty (A) and epinephrine-loaded (C) nanoparticles, diameter size distributions analyzed by zetasizer of empty (B) and epinephrine-loaded nanoparticles (D), and TEM of nanoparticles after 6 weeks of epinephrine release (E).

generated using a double emulsion solvent evaporation technique to encapsulate water-soluble molecules. Physical characterization of POE nanoparticles was evaluated using various water-soluble molecules such as, epinephrine, rhodamine 6G, and BSA with molecular weights of 333, 479, and 66 000 g/mol, respectively. Cytotoxicity and nanoparticle cell uptake studies were also evaluated in detail.

3.1. Physico-Chemical Characterization of POE Nanoparticles. Table 1 summarizes the main physicochemical characteristics of the POE nanoparticle formulations. All POE nanoparticles displayed average size ranges from 241 to 298 nm. Epinephrine-loaded nanoparticles were smaller than empty nanoparticles. This size discrepancy may be attributed to the stabilization of the primary W/O emulsion in the presence of epinephrine. This stabilization reduces coalescence of droplets and leads to smaller particle sizes.[30] The epinephrine-induced stabilization is further evidenced by the low polydispersity values. Epinephrine-loaded nanoparticles had a lower PDI (≤0.112) than empty nanoparticles (≤0.25). In fact, in all cases, the particle size distribution was quite homogeneous showing low PDI values (≤0.25). There was no significant effect of POE molecular weight on nanoparticle size. However, by changing the type of encapsulated water-soluble molecule from small molecules to a large molecule, an increase in particle size was observed. All nanoparticles were anionic in nature, and the zeta potential changed according to the net charge of the active agent taken for encapsulation. Specifically, the decrease in the negative charge of epinephrine nanoparticles is likely due to the cationic nature of epinephrine, while the high negative zeta potential of BSA nanoparticles may be due to its anionic nature. The yield of the nanoparticles ranged from 66.8% to 73.9%, confirming that the majority of the polymer was converted into nanoparticles. Encapsulation efficiencies of drug/dye-loaded nanoparticles, ranged from 21.4% to 63.3%. A higher loading was observed with the large, water-soluble molecule, BSA, in comparison with a small water-soluble drug/dye. Generally, high encapsulation efficiency with water-soluble molecules is a difficult task to achieve, due to the rapid diffusion of water-soluble drugs from the hydrophobic organic phase into the external aqueous phase that takes place during the preparation of nanoparticle emulsions.[31] In the present study, BSA, being large in size, diffused slowly from the internal aqueous phase, and as a result, the highest encapsulation efficiency was achieved with this molecule.

Figure 2 shows the morphological analysis by electron microscopy of empty and epinephrine-loaded nanoparticles. TEM studies demonstrated the spherical shape of both nanoparticles. Capsular structures with homogeneous shells were visible in both empty and epinephrine-loaded POE nanoparticles. With the presence of an exterior hydrophobic POE polymer shell formation on the polymer matrix core, these POE nanoparticles were integrated as nanocapsules.[32] Epinephrine was presented in a polymer matrix core and protected by polymeric shell. Figure 2E depicts the TEM image of the epinephrine-loaded POE nanoparticle after 6 weeks of epinephrine delivery. Even after 6 weeks of time, nanoparticles remained nearly spherical in shape and retained a POE shell, similar to fresh POE nanoparticles. The average diameter of nanoparticles as visualized by TEM fell within the particle size distribution range determined by DLS (Figure 2B,D).

3.2. In Vitro Epinephrine Release Studies. In general, the molecular weight of the polymer is one of the key factors affecting the drug release. Molecular weight is indicative of the chain length of the polymer, and the higher the molecular weight, the longer the chain length. Furthermore, chain length reflects the hydrophilicity/lipophilicity of the polymer. An increase in chain length increases the lipophilicity and decreases the degradation rate of the polymer. Therefore, by varying the molecular weight, the degradation rate of the polymer and release kinetics of the drug can be controlled accordingly.

Understanding the degradation phenomenon of POE is important, as it determines the rate and mechanism of release of the therapeutic agent. POE undergoes surface erosion through heterogeneous hydrolytic erosion, which confines the hydrolysis to the surface of the drug delivery system, which is commonly referred to as surface erosion.[21] This process is capable of giving rise to a zero-order drug release for drug delivery system with a constant surface area. The acidic (lactic acid) monomer and oligomers thus formed further catalyze the degradation of the parent polymer, a process known as autocatalysis.[21] The release of entrapped therapeutic agent from POE polymer has been found to occur through a degradation-mediated process.[24−26] However, the literature lacks any inclusive report describing the influence of molecular weight on release behavior from POE nanosized delivery systems. Hence, this article presents, for the very first time, POE nanoparticles synthesis and characterization with the goal of designing a drug delivery carrier to treat various intraocular diseases.

Increasing the molecular weight from 5 to 22 kDa significantly decreased the release rate of epinephrine from POE nanoparticles ($p > 0.0001$). Nanoparticles of 5 kDa and 22 kDa molecular weight released 29.5% and 20.4%, respectively, of total drug within 14 weeks at a nearly constant rate (Figure 3A). The cumulative drug release was fitted into various release models, namely, zero-order, first-order, Higuchi's square root plot, and Hixson−Crowell cube root plot. The model giving a correlation coefficient close to unity was taken as the correct method of release. Zero-order release patterns were observed for both POE nanoparticles of 5 and 22 kDa molecular weight, with R^2 values of 0.998 and 0.999, respectively.

It is known that POE polymer serves a good model for the chemically controlled drug release mechanism.[24−26] Degradation plays a dominant role in controlling the release rate of encapsulated molecules. The rate of degradation is not constant and increases continuously with time. This keeps the release

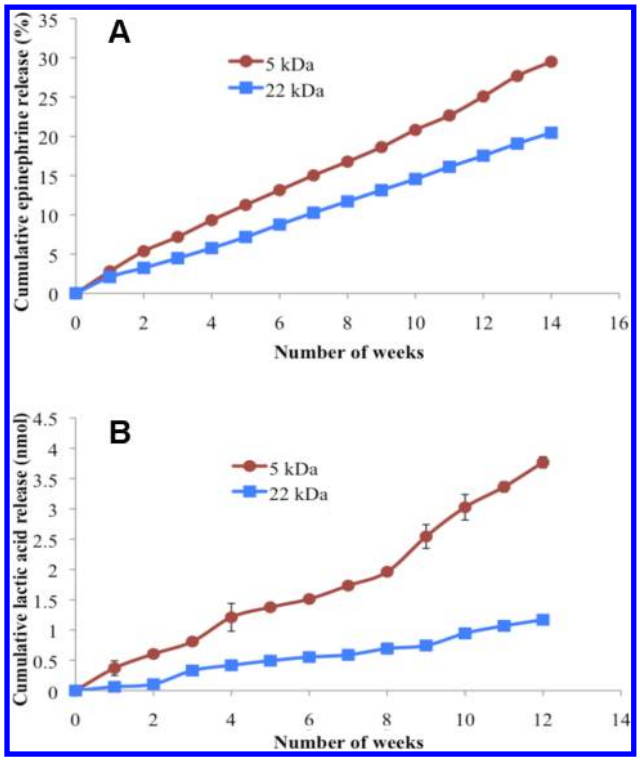

Figure 3. In vitro release profiles of epinephrine-loaded nanoparticles (A) and lactic acid release from empty POE nanoparticles (B) in pH 7.4 phosphate buffer. Data shown as mean ± standard error ($n = 3$).

rate constant (zero-order), which otherwise tends to decrease due to diffusion (a concentration dependent process). An increase in degradation rate with time can be justified on the basis of two factors, namely, autocatalysis and glass transition temperature of the polymer (T_g). Acidic monomers, lactic acid, formed during degradation further catalyze the degradation and increase the degradation rate with the time. The glass transition temperature depends on the polymer molecular weight, and under typical conditions, a decrease in molecular weight lowers the T_g, which determines a glassy and a rubbery state of the polymer below and above it. In the case of low molecular weight POE nanoparticles, rapid water hydration, due to less lipophilicity of the polymer, allows the T_g to shift to the lower temperature. This phenomenon is called the plasticizing effect of the water, and it makes polymer chain segments more mobile and thus more labile for degradation.[33] DSC studies revealed that the T_g values for 22 and 5 kDa molecular weight POE polymers were −14 °C[27] and −19.96 °C, respectively. Thus, there is a decrease in T_g observed with the decrease in molecular weight in our polymer.

Also, a difference in the release rate can be attributed to the difference in encapsulation efficiencies. In the case of POE nanoparticles derived from 22 kDa molecular weight polymer, the encapsulation efficiency was lower (~21.4%) compared to formulation with 5 kDa molecular weight POE nanoparticles (~31.6%). Thus, to obtain the same amount of drug release from 5 kDa molecular weight POE nanoparticles, a larger quantity of nanoparticles of 22 kDa molecular weight polymer had to be used. This proportionate increase in the polymer may have been reflected in sustaining the release to a greater extent in the formulation with 22 kDa molecular weight POE nanoparticles.

In addition to its molecular weight, the degradation behavior of a nanoparticulate system also depends on the hydrophobic nature of the polymer. Specifically, the more hydrophobic the polymer, the slower its degradation. The hydrophobicity of the polymer is influenced by the ratio of amorphous to crystalline regions, which in turn is determined by the latent acid group [lactic acid or glycolic acid]. Lactic acid, being more hydrophobic than glycolic acid, makes POE with D,L-lactide more hydrophobic and subsequently slows down the degradation process.[21,23] Therefore, by adjusting the lactide/POE ratio, the desired degradation rate and, subsequently, release pattern of the drug can be achieved.[34]

Most drug-loaded particulate formulations show a biphasic release pattern wherein there is an initial burst followed by a sustained release.[35] The high initial release may be due to the presence of free and weakly bound drug on the surface of particulate carriers. However, no such initial burst release was observed in the present case, possibly suggesting the absence of any unbound drug associated with the particles (Figure 3A). Even though it is a difficult task to design a long-term, zero-order release study for nanoparticle formulations due to a number of practical problems associated with it, POE nanoparticles are in an excellent position to serve as an ideal drug carrier for intraocular therapies.

3.3. In Vitro Biodegradation of POE Nanoparticles. In a slow eroding POE polymer, the degradation process is initiated by the breakdown of dilactate bonds, which simultaneously release free lactic acid. These acid fragments further catalyze ortho ester cleavages and results in the production of propionic acid.[21] In the same way, POE nanoparticles also release lactic acid, which indicates the initiation of POE nanoparticle degradation (Figure 3B). We measured no lag period before lactic acid was released from POE nanoparticles (Figure 3B). In contrast, with POE polymer, there was approximately 20 days of lag time to break down the dilactate bonds.[21] This can be explained by the increase in surface area of POE nanoparticles in comparison with bolus polymer. The most significant observation is that lactic acid was released from both nanoparticles of 5 and 22 kDa molecular weight POE, linearly for the entire 12 week period of time. The cumulative drug release was fitted into various release models, namely, zero-order, first-order, Higuchi's square root plot, and Hixson–Crowell cube root plot. The model giving a correlation coefficient close to unity was taken as the correct method of release. Zero-order release patterns were observed for both POE nanoparticles of 5 and 22 kDa molecular weight, with R^2 values of 0.976 and 0.981, respectively (p = 0.0002). Nanoparticles of 5 kDa POE released 3.77 nmol, whereas nanoparticles of 22 kDa POE released 1.17 nmol of lactic acid. These values clearly reflect the degradation rate of both 5 and 22 kDa molecular weight POE nanoparticles. POE nanoparticles of 5 kDa molecular weight have a faster degradation than 22 kDa molecular weight. It is known from POE polymers that the rate of degradation is directly related to the concentration of lactic acid release, and as a result, encapsulated drug release is observed.[21] POE nanoparticles also follow the same trend in lactic acid release and epinephrine release, which reflects POE nanoparticle degradation. TEM images (Figure 2) demonstrate that there is a subtle change in surface morphology between fresh (Figure 2C) and 6 week old nanoparticles (Figure 2E). One can see few layers around nanoparticles after 6 weeks of release, whereas there were no such layers observed around fresh nanoparticles.

3.4. In Vitro Cytotoxicity of Nanoparticles. Potential cytotoxicity of nanoparticles was evaluated in vitro using an MTT assay on HEK-293 cell lines. Our results (Figure 4) showed that nanoparticles generated using both 5 and 22 kDa molecular weight polymers have low cytotoxicity. The relative cell viability when exposed to all nanoparticle concentrations was between 84 and 100% viability, with the exception of 1 mg/mL nanoparticles. Cells exposed to 1 mg/mL showed 65% and 77% cell viability when exposed to nanoparticles made from 5 and 22 kDa molecular weight polymers, respectively. Cell viability of positive controls, 1% Triton-X 100 and 80 μm H_2O_2 measured as 5% and 8% viable, respectively. Cell culture medium served as a negative control, which showed 100% cell viability.

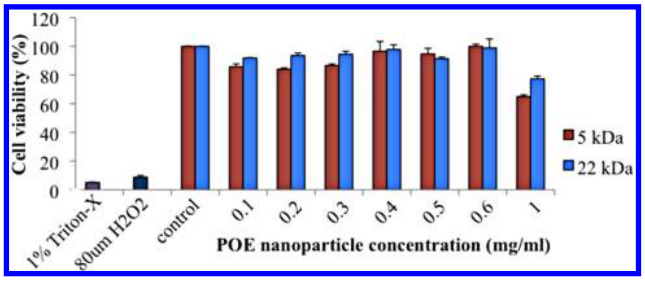

Figure 4. Cytotoxicity of nanoparticles of 5 and 22 kDa molecular weight POE. Data shown as mean ± standard error (n = 3).

3.5. POE Nanoparticle Uptake by Cells. *3.5.1. Qualitative.* Rhodamine 6G-loaded nanoparticles were used to qualitatively evaluate the potential uptake of nanoparticles into both Müller cells (Figure 5) and HEK-293 (data not shown). Figure 5 illustrates representative confocal images of Müller cells exposed to rhodamine 6G-loaded nanoparticles under various conditions. Cells were exposed to rhodamine 6G-loaded nanoparticles, washed only once, and imaged to determine if nanoparticles adsorbed to the cell membranes or were internalized. Our data shows that the vast majority of the rhodamine 6G-loaded nanoparticles were washed away and did not adsorb to the membranes of the cells irrespective of the concentration of nanoparticles and incubation times used. Of the few nanoparticles that did adsorb, none were internalized by either Müller or HEK-293 cells. Eun Chul Cho et al. observed that neutral and anionic nanoparticles were adsorbed to a lesser degree to the negatively charged cell-membrane surface and consequently show lower levels of internalization as compared to cationic nanoparticles.[36] In our present study, rhodamine 6G-loaded POE nanoparticles were anionic with −15.8 mV surface charge. In addition, both Müller cells and HEK-293 have negatively charged cell membrane surfaces.[37,38] Because of this, rhodamine 6G-loaded POE nanoparticles had very little affinity with either cell line. Unlike cancer therapies, intraocular drug delivery systems should remain extracellular to deliver drugs for prolonged periods so as to avoid repeated intraocular injections.

3.5.2. Quantitative. To further confirm the rhodamine 6G-loaded nanoparticle cell uptake data obtained from confocal images, rhodamine 6G was quantified in both Müller cell and HEK-293 uptake studies, under various experimental conditions (Tables 2 and 3). The amount of rhodamine 6G was quantified in all three washings and cell lysates of both Müller cells and HEK-293. In all cases, most of the rhodamine 6G was observed preferentially in wash I, while only a small quantity

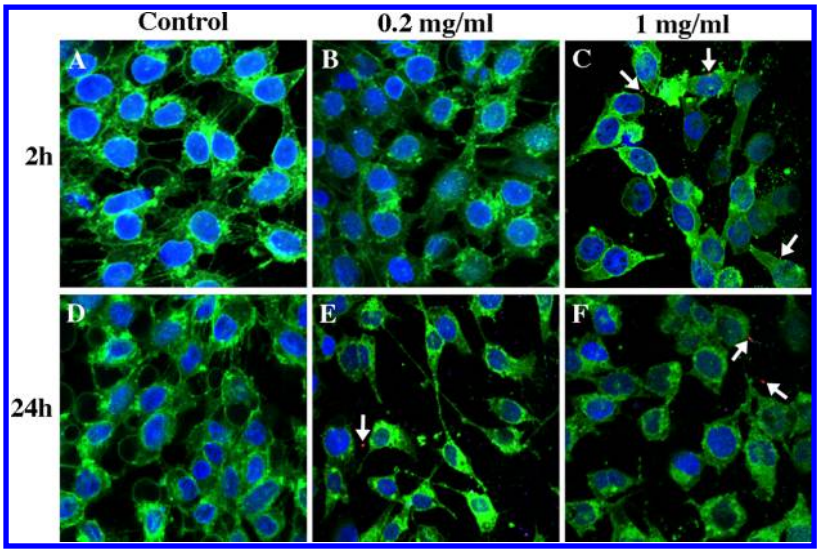

Figure 5. Confocal fluorescence microscopy images of Müller cells after exposure to nanoparticles. Cell membranes and nuclei were labeled with Oregon Green 488 DHPE and To-Pro-3 iodide and are shown in green and blue, respectively. POE nanoparticles loaded with rhodamine 6G are represented with red color and shown with white arrows.

Table 2. Quantification of Rhodamine 6G in Müller Cell Uptake Studies

sample (mg/mL)	incubation time	wash I[a]	wash II[a]	wash III	cells
0.2	2 h	93.2% ± 0.05	n/d	n/d	n/d
1	2 h	94.0% ± 0.14	1.90% ± 0.01	n/d	n/d
0.2	24 h	96.1% ± 0.05	0.52% ± 0.03	n/d	n/d
1	24 h	95.4% ± 0.16	0.80% ± 0.01	n/d	n/d

[a]Mean ± standard error (n = 3). n/d denotes not in a detectable range.

Table 3. Quantification of Rhodamine 6G in HEK-293 Cell Uptake Studies

sample (mg/mL)	Incubation time	wash I[a]	wash II[a]	wash III	cells
0.2	2 h	90.5% ± 0.13	4.60% ± 0.03	n/d	n/d
1	2 h	93.2% ± 0.22	n/d	n/d	n/d
0.2	24 h	92.4% ± 0.14	2.80% ± 0.11	n/d	n/d
1	24 h	92.7% ± 0.062	0.12% ± 0.03	n/d	n/d

[a]Mean ± standard error (n = 3). n/d denotes not in a detectable range.

was present in wash II. Rhodamine 6G was beyond the limits of detection in both wash III and cell lysates. These quantitative data strongly support our qualitative data, and confirm that POE nanoparticles were not internalized irrespective of their concentrations, cell lines, and incubation time periods.

4. CONCLUSIONS

In summary, we successfully prepared POE nanoparticles using a double emulsion solvent evaporation method. Moreover, we were able to encapsulate a variety of water-soluble molecules such as epinephrine and rhodamine 6G as a representation for small molecules and BSA as a representation for large molecule. The current investigation demonstrates that prolonged controlled release can be tailored significantly by varying the POE polymer molecular weight. Furthermore, POE nanoparticles have a significant role in enhancing the delivery aspects of water-soluble small drug molecules like epinephrine. Epinephrine release followed perfect zero-order kinetics throughout a period of 14 weeks. By increasing the molecular weight of the polymer, degradation of POE nanoparticles slowed down, and simultaneously, drug release slowed down. In vitro cytotoxicity data showed that nanoparticles at concentrations up to 1 mg/mL were very well tolerated by HEK-293 cells. POE nanoparticle cell uptake studies qualitatively and quantitatively confirmed that particles were not taken up by either Müller cell or HEK-293 lines, which is also independent of the concentration of the nanoparticles and incubation time. Thus, POE nanoparticles have all of the characteristics of an efficient drug delivery carrier by improving the intraocular bioavailability and decreasing the dosing frequency, thereby minimizing the dose-dependent adverse effects and maximizing the patient compliance. In vivo intravitreally injected POE nanoparticle distributions and toxicity experiments in rabbits are in progress.

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Notes

The authors declare no competing financial interest.

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