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RESEARCH PAPER

Novel Topical Ophthalmic Formulations for Management of Glaucoma

Mohammed M. Ibrahim • Abd-Elgawad H. Abd-Elgawad • Osama A. Soliman • Monica M. Jablonski

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ABSTRACT

Purpose Preparation of topical ophthalmic formulations containing brimonidine-loaded nanoparticles prepared from various biodegradable polymers—PCL, PLA and PLGA—for sustained release of brimonidine as a once daily regimen for management of glaucoma.

Methods Nanoparticles were prepared using spontaneous emulsification solvent diffusion method then characterized regarding their particle size, zeta potential, morphology and drug contents. Brimonidine-loaded nanoparticles were incorporated into eye drops, temperature-triggered *in situ* gelling system and preformed gel and characterized regarding their pH, viscosity, uniformity of drug contents, *in vitro* release study, *in vitro* cytotoxicity and *in vivo* intraocular pressure (IOP) lowering effects.

Results The results of optimized brimonidine-loaded PCL-, PLGA- and PLA-NPs respectively, are: particle sizes of 117.33 ± 4.58 nm, 125.67 ± 5.15 nm and 131.67 ± 3.79 nm; zeta potentials of -18.5 ± 2.87 mV, -21.82 ± 2.7 mV and -28.11 ± 2.21 mV; and encapsulation efficiencies of $77.97 \pm 1.38\%$, $68.65 \pm 3.35\%$ and $73.52 \pm 2.92\%$. TEM analyses revealed that all NPs have spherical shapes with dense core and distinct coat. *In vitro* release data showed a sustained release without any burst effect with Higuchi non-Fickian diffusion mechanism. Cytotoxicity studies revealed that all formulations are non-toxic. Also all formulations possessed a sustained IOP lowering effect compared to Alphagan® P eye drops.

Conclusions Our formulations showed prolonged management of glaucoma that should meet with better patient compliance as a once-daily formulation.

M. M. Ibrahim • M. M. Jablonski (✉)
Department of Ophthalmology, Hamilton Eye Institute
University of Tennessee Health Science Center
930 Madison Avenue, Suite 731, Memphis, Tennessee 38163, USA
e-mail: mjablonski@uthsc.edu

M. M. Ibrahim • A.-E. H. Abd-Elgawad • O. A. Soliman
Department of Pharmaceutics, Faculty of Pharmacy
Mansoura University, Mansoura 35516, Egypt

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ABBREVIATIONS

%DL	Drug loading
%EE	Encapsulation efficienc
%Y	Yield
AUC_{rel}	Relative area under IOP *versus* time curve to the control preparation
AUC_{total}	Total area under IOP *versus* time curve
DCM	Dichloromethane
DIW	De-ionized water
DMSO	Dimethyl sulfoxide
EMEM	Eagle's minimal essential medium
HPMC	Hydroxypropylmethylcellulose
I_{max}	Maximum decrease in IOP
IOP	Intraocular pressure
MC	Methylcellulose
MTT	Methyl thiazol tetrazolium
NPs	Nanoparticles
PBS	Phosphate buffered saline
PCL	Poly-ε-caprolactone
PDI	Polydispersity index
PLA	Poly(L-lactide)
PLGA	Poly(D,L-lactide-co-glycolide)
PVA	Polyvinyl alcohol
TEM	Transmission electron microscope
T_{end}	Time required for IOP to return again to its baseline
T_{max}	Time required to reach maximum decrease in IOP

INTRODUCTION

Brimonidine is a potent and relatively selective α_2-adrenergic receptor agonist that can be effectively used in management of open-angle glaucoma. It has the ability to reduce intraocular

pressure (IOP) by decreasing the production of aqueous humor and by increasing its uveoscleral outflow (1). The drug is available in two chemical forms that differ in their solubility—brimonidine tartrate salt which has a good water solubility (34 mg/ml) and brimonidine free-base which has a negligible water solubility (2). Brimonidine is commercially available as eye drops that contain 0.1 or 0.15% brimonidine tartrate salt solution (Alphagan® P, Allergan Inc., Irvine, CA), which provides efficient management for glaucoma but it suffers from poor patient compliance as it requires several dosing per day due to its short pre-corneal retention time (3). The preparation of brimonidine in the form of a biodegradable sustained release topical formulation would be advantageous because it can be used once daily. Several studies have attempted to prepare brimonidine sustained release preparations to increase its pre-corneal retention time and decrease its dosing frequency, including brimonidine tartrate-containing Eudragit NPs (4), brimonidine tartrate biodegradable and bioadhesive ocular inserts (3), brimonidine tartrate chitosan NPs (5), and a brimonidine polyamidoamine dendrimer hydrogel (2). However, none of these studies was able to achieve an extended release ophthalmic dosage form of sufficient duration. More recently Yang *et al.* reported a novel sustained release hybrid hydrogel/PLGA-NPs platform for glaucoma management in which the NPs containing brimonidine and timolol maleate combination (6). In our study, we sought to formulate sustained release brimonidine topical ophthalmic formulations to be administered non-invasively once daily for treatment of glaucoma.

Glaucoma is a significant public health problem worldwide (7). Although high IOP is not the only risk factor associated with glaucoma, most of the current treatment strategies, such as medication or surgery, focus on lowering IOP. Downstream of IOP elevation, retinal ganglion cell degeneration leads to irreversible blindness in this disease. Studies have demonstrated that brimonidine can also promote survival of the injured retinal ganglion nerve cells by activation of the α_2-adrenergic receptors in the retina and/or optic nerve. Therefore, brimonidine is also a neuroprotective agent that can prevent further damage of the injured neurons in several models of ischemia and glaucoma (8). Because of this dual action, brimonidine is considered a highly useful therapeutic approach in glaucoma management in which both neuroprotection and IOP reduction are required outcomes of the therapeutic regimen (9).

Nanocarrier design is a very attractive aspect of modern drug delivery research due to its ability to deliver drugs to the right place, at the appropriate time and at accurate concentrations. Nanocarriers are nanoparticulate systems that entrap drugs that are intended for administration by several routes. Nanoparticules (NPs) may prevent or minimize drug degradation and metabolism and increase cellular uptake (10). In addition, they have many advantages such as having a long shelf life, being made from safe materials including synthetic and natural biodegradable polymers, lipids and polysaccharides, and having the ability to pass important mucosal barriers, such as the intestinal, nasal and ocular barriers (11).

A major drawback of conventional topical ophthalmic drug delivery systems is the rapid and extensive loss of drug caused by the drainage through the nasolachrymal duct and high tear fluid turnover (12). Several studies have attempted to increase the corneal penetration of drugs using colloidal drug delivery systems, using liposomes (13), NPs (14) and nanocapsules (15), which in turn improve its therapeutic effect. Another approach is to use polymers to prolong the release of drug. Poly-ε-caprolactone (PCL), poly(L-lactide) (PLA), and poly(D,L-lactide-co-glycolide) (PLGA) are biodegradable and biocompatible polymers that are approved by the US-FDA (16). Chemically, they are polyesters that are degraded by the cleavage of the ester bonds in an aqueous system yielding non-toxic biocompatible degradation products (16,17).

In the present study, we use our recently developed a modified spontaneous emulsification solvent diffusion method (18) to prepare brimonidine-loaded nanoparticles prepared from different synthetic biodegradable polymers including PCL, PLA and PLGA for topical ophthalmic use. We then incorporated the drug-loaded nanoparticles in three different ophthalmic dosage forms including eye drops, a temperature triggered *in situ* gelling system and a preformed gel. All formulations are biocompatible and provide prolonged reduction in IOP.

MATERIALS AND METHODS

Materials

Brimonidine (U104, UK 14304), PCL (average molecular weight 14,000 Da), PLA (molecular weight 152,000 Da), PLGA (lactide:glycolide 75:25, molecular weight 66–107 kDa), hydroxypropylmethylcellulose (HPMC), methylcellulose (Methocel, MC), polyvinyl alcohol (PVA, molecular weight 31–50 kDa), Triton X-100, methyl thiazol tetrazolium (MTT), sodium chloride, potassium chloride, sodium phosphate dibasic, potassium dihydrogen phosphate, absolute ethyl alcohol, acetone and dichloromethane (DCM) were purchased from Sigma-Aldrich (St. Louis, MO). Soybean L-α-Lecithin (98% phosphotidyl choline) was purchased from Calbiochem (San Diego, CA). Poloxamer 188 (Pluronic F68; Polyethylene-Polypropylene Glycol, block copolymer of ethylene oxide and propylene oxide, average molecular weight 8,400 Da) was purchased from Spectrum

Chemical Mfg. Corp., (New Brunswick, NJ). Dimethyl Sulfoxide (DMSO) was purchased from ThermoScientific Co. (Rockford, IL). Glacial acetic acid was purchased from Fisher Scientific (Fair Lawn, NJ). Eagle's minimal essential cell culture medium (EMEM) was purchased from ATCC Co. (Manassas, VA). Isothesia (Isoflurane USP) was purchased from Butler Schein Animal Health (Dublin, OH). All chemicals utilized for preparing buffers are of the analytical grade. All materials were used as received without any further treatment.

Animals

Two lines of BXD mice—BXD29 and BXD96—were obtained from Dr. Robert Williams (Department of Anatomy and Neurobiology, UTHSC, Memphis, TN). These mice were selected after clinically screening our large family of BXD recombinant inbred lines of mice. Both lines were selected because they harbor wildtype alleles of *Tyrp1* and *Gpnmb* (19,20) and they do not develop pigmentary dispersion glaucoma (21). BXD29 has spontaneously elevated IOP, while BXD96 has an IOP in the normal range. The mice were aged 3–5 months and weighed 25–35 g. They were housed in standard cages in an air-conditioned and light-controlled room (12 h light and 12 h dark cycles) at 25±2°C and at 70±5% relative humidity. They had a free and continuous access to food and water. All mice met the following criteria: 1) they were healthy and free of any clinically observable abnormalities; 2) both the eyes were healthy with no injury or history of injury; 3) the basal IOP ranged 18±2 mmHg for BXD29 and 15±2 mmHg for BXD96; and 4) the IOP difference between eyes of the same mouse did not exceed 2 mmHg. All experimental protocols were approved by the Animal Care and Use review board of the University of Tennessee Health Science Center. Mice were handled in a manner consistent with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, the Public Health Service Policy on Humane Care and Use of Laboratory Animals).

Preparation of Plain and Brimonidine-Loaded Nanoparticles

Plain nanoparticles were prepared using a spontaneous emulsification solvent diffusion technique using our published protocols (18). Briefly, polymer and lecithin were dissolved in DCM/acetone mixture to give the organic phase which was injected into the aqueous solution of stabilizer (PVA or poloxamer 188) under magnetic stirring. The mixture was sonicated then stirred overnight to evaporate the organic solvent. Nanoparticles were separated by ultracentrifugation and washed several times prior to lyophilization. To optimize our formulation, we studied the effect of several formulation parameters on final particle size, polydispersity index (PDI) and zeta potential. The variables we tested include: the type of polymer and its concentration; the type of stabilizer and its concentration; and the concentration of emulsifier (lecithin). Our criteria for selecting the optimum formula for each polymer included a small and consistent particle size and a high zeta potential. These formulations were further evaluated with regard to the effect of acetone in the organic phase, using DCM as a control. The effect of various sonication times (i.e., 0, 5, 10, 15 min) and the effect of lyophilization on the particle size was studied by measuring it before and after lyophilization keeping all other parameters constant. Brimonidine-loaded NPs was prepared by the same method by dissolving brimonidine in the organic phase at a concentration of 0.05%.

Measurement of Particle Size, Zeta Potential and Morphology

NPs were characterized using our published protocols (18). The evaluation consisted of measurement of particle size and zeta potential using a zetasizer (Nanoseries, Nano-ZS, Malvern Instruments Limited, UK) after suitable dilution by DIW. Statistical analyses of variations in particle size and zeta potential data were performed using unpaired t-tests and GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA). All experiments were performed in triplicate. The shape and surface morphology of NPs were examined with a transmission electron microscope (TEM) (JEM-2000EX II Electron Microscope, JEOL, LTD, Tokyo, Japan), after applying diluted solution of NPs on a formvar coated copper grid and drying in a dessicator, as described previously (18).

Drug Loading Assessment

To determine the amount of brimonidine incorporated into the NPs, 5 mg of brimonidine-loaded NPs were weighed and dissolved in 2 ml DMSO in a 25 ml volumetric measuring flask and brought up to volume by absolute ethyl alcohol. The solution was assayed spectrophotometrically at 261 nm (μ-Quant Universal Microplate Spectrophotometer Bio-Tek Instruments, Inc., USA). The total drug content of each formulation was calculated from a standard curve (with a linearity coefficient (r)=0.999). After controlling for any absorbance due to blank NPs, all measurements fell within the calibration linearity range of the calibration curve. Each experiment was repeated three times. The encapsulation efficiency (% EE),

drug loading (% DL) and NP yield (% Y) were calculated using the following equations.

$$\%\ \mathrm{EE} = \frac{\text{Weight of brimonidine entrapped within nanoparticles}}{\text{Total brimonidine added}} \times 100$$

$$\%\ \mathrm{DL} = \frac{\text{Weight of brimonidine entrapped within nanoparticles}}{\text{Total weight of nanoparticles}} \times 100$$

$$\%\ \mathrm{Y} = \frac{\text{Total weight of nanoparticles}}{\text{Weight of polymer} + \text{weight of brimonidine}} \times 100$$

Preparation of Topical Ophthalmic Formulations

Plain ophthalmic dosage forms—eye drops, a temperature-triggered *in situ* gelling system, and a preformed gel—were prepared using our published methods (18). Briefly, eye drops were prepared by dissolving 2% w/v HPMC in PBS and the *in situ* gelling system was prepared by dissolving 3% w/v HPMC and 17% w/v poloxamer 188 in PBS. The preformed eye gel was prepared by dissolving 4.5% w/v MC in PBS. Chemicals used for the preparation of plain ophthalmic formulations and their ratios were selected after preliminary experiments to determine the optimal conditions that gave clear and transparent formulation with suitable consistency. The composition of the *in situ* gelling system was optimized after testing several prepared *in situ* gelling system formulations by dropping them in an artificial tears solution at 35°C ± 0.5 and observing the clarity of the formed gel, gelation time and the time taken for the formed gel to dissolve (18,22). Brimonidine-loaded NPs were then incorporated in the plain ophthalmic dosage forms to give a final concentration of brimonidine equivalent to 0.1% w/v. The pH of each formulation was determined using a pH meter (Corning pH meter 440, Corning Incorporated, Corning, NY) after dilution in DIW (18).

Characterization of Topical Ophthalmic Formulations

The viscosity of each formulation was determined using a cone (1.5°) and plate rotary viscometer (Brookfield DV-II+ programmable viscometer, Brookfield Engineering Laboratories INC., USA) at 35°C ± 0.5 using our published protocols (18). To study temperature-induced gelation, measurements were also performed at 25°C ± 0.5 using the *in situ* gelling systems. Each experiment was repeated three times and the results were calculated as mean ± SD.

The uniformity of drug content in each formulation was determined using our published methods (18). Briefly, 1 g of each formulation was dissolved in DMSO and diluted with absolute ethyl alcohol. After filtering, the clear solution was assayed spectrophotometrically for its drug content with appropriate negative controls. The experiments were repeated three times and the results were calculated as mean ± SD.

The release of brimonidine from our drug-loaded NP formulations containing was performed using Fast Micro-Equilibrium Dialyzers (Harvard Apparatus Co., Holliston, MA) with chamber volume 1,500 μl and membrane molecular weight cut-offs of 3,500, 15,000 and 25,000 Da, as previously described (18). Briefly, 100 mg of each ophthalmic formulation containing brimonidine-loaded NPs or 0.1% Alphagan® P eye drops was placed in the donor chamber and warmed PBS was placed in the acceptor chamber. The dialyzer was kept in thermostatically controlled shaker (35°C ± 0.5 and 50 rpm). The released amount of brimonidine in the withdrawn samples of each formulation was analyzed spectrophotometrically for its drug contents. Control experiments were performed using plain NPs incorporated in plain dosage form vehicles. All experiments were repeated three times and the concentrations were calculated from standard curves. Release data were statistically analyzed using one-way ANOVA followed by Tukey-Kramer multiple comparisons tests (23). Statistical calculations were carried out using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA).

The *in vitro* cytotoxicity of brimonidine (0.1%), Alphagan® P and our formulations were evaluated by an MTT assay and HEK293 cells by incorporation of the formulations with the HEK293 cell line for 24 h then measuring the cell viability by testing the ability of the cell line to reduce the yellow MTT to its purple formazan which can be measured spectrophotometrically at 570 nm (18). Statistical analysis of the percent cell viability data was performed using a one-way ANOVA with Tukey- Kramer multiple comparison tests (23). Each experiment was done in six replicates.

In Vivo Efficacy of the Ophthalmic Formulations

The efficacy of the ophthalmic formulations was determined using a single dose response design. Mice ($n=5$) from each BXD strain were lightly sedated using isoflurane inhalation. Ten microliters of the ophthalmic formulations containing PCL-, PLA- or PLGA-NPs or the control preparation (i.e. Alphagan® P) was instilled into the inferior conjunctival sac of the right eyes of mice, while the left eyes served as control. Eyes were held open for at least 20 s then the two eyelids were held together for an additional 10 s to allow for adequate ocular surface contact of the ophthalmic formulations, and to prevent aggressive blinking during application of the drug. The intraocular pressure of both eyes was measured using a Tonolab tonometer (Colonial Medical Supply, Franconia, NH) immediately before the application of each formulation (to establish the baseline of both eyes) and at various time intervals after application until the IOP

Table I Composition of Brimonidine-Loaded Nanoparticles

Ingredient (% w/v)	PCL-NPs	PLA-NPs	PLGA-NPs
Brimonidine	0.05	0.05	0.05
PCL	0.1	–	–
PLA	–	0.1	–
PLGA	–	–	0.1
Lecithin	1	1	1
Poloxamer 188	0.5	–	–
PVA	–	0.5	0.5
Acetone	4	4	4
DCM	8	8	8
DIW to	100	100	100

PCL poly-ε-caprolactone, *PLA* poly(L-lactide), *PLGA* poly(D,L-lactide-co-glycolide), *PVA* polyvinyl alcohol, DCM: dichloromethane, *DIW* deionized water

returned to baseline. All measurement periods began at the same hour of each day (8 am). The IOP measurements were taken three times for each eye at each time interval.

Evaluation of the ophthalmic formulations was based on comparing pharmacodynamic parameters including: maximum decrease in IOP (I_{max}); the time required to reach maximum decrease in IOP (T_{max}); the time required for IOP to return again to its baseline (i.e. end of drug effect) (T_{end}); the total area under IOP *versus* time curve (AUC_{total}); and the relative area under IOP *versus* time curve (AUC_{rel}), which can be estimated from the quotient of the values of AUC_{total} of the ophthalmic formulations and that of the control preparation. AUC_{total} values were determined using the linear trapezoidal method. All results were reported as mean ± SEM. Statistical analyses of the results were performed using one-way ANOVA with Tukey-Kramer multiple comparisons tests (23). Calculations of all pharmacodynamic parameters as well as the statistical analysis of the results were calculated using GraphPad Prism-5 software (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Characterization of Brimonidine-Loaded Nanoparticles

The optimized NPs formulations that had the smallest particle size and the highest zeta potential for PCL contained 0.1% PCL, 1% lecithin and 0.5% poloxamer 188, while for PLA and PLGA they contained 0.1% polymer, 1% lecithin and 0.5% PVA (Table I). After incorporation of brimonidine, there was a non-significant increase in the mean particle size and a non-significant decrease in the negative zeta potential of all NPs compared to the plain NPs ($p > 0.05$, Table II). Moreover, the encapsulation efficiency of brimonidine was high (77.97, 73.52 and 68.65% for PCL-, PLA- and PLGA-NPs, respectively) with a final drug loading of 27.46, 26.21 and 23.89% for PCL-, PLA- and PLGA-NPs, respectively. The brimonidine content of the prepared ophthalmic formulations was in accordance with the USP official requirements as the deviation of the drug contents from the originally added active constituents are less than ±3% (24). Also the small standard deviations we achieved indicate a uniform distribution of the brimonidine-loaded NPs within the ophthalmic formulations vehicles. TEM analyses confirm the particle size data obtained by the zetasizer. In addition, the NPs have a distinct shape which is spherical with a solid dense polymer core surrounded by evenly distributed coat that may be comprised of lecithin and NPs stabilizer (PVA or poloxamer 188). Drug incorporation did not affect the shape of the nanoparticles (Fig. 1).

In Vitro Evaluation of the Formulations

Presented in Table III are the pH, drug content as well as the viscosities at 35°C and 10 rpm of our ophthalmic formulations. The pH of our ophthalmic formulations ranged from 7.73 to 7.84 and the drug content ranged from 98.78% to

Table II Characteristics of Plain and Brimonidine-Loaded NPs

Parameters		Evaluation (mean ± SD)		
		PCL-NPs	PLA-NPs	PLGA-NPs
Particle size (nm)	[a]Plain	113.67 ± 6.16	122.33 ± 4.53	119.33 ± 4.58
	Drug loaded	117.33 ± 4.58	131.67 ± 3.79	125.67 ± 5.15
PDI	[a]Plain	0.231 ± 0.01	0.202 ± 0.015	0.215 ± 0.011
	Drug loaded	0.26 ± 0.002	0.27 ± 0.008	0.21 ± 0.029
Zeta potential (mV)	[a]Plain	−21.65 ± 3.20	−30.70 ± 2.38	−24.51 ± 3.13
	Drug loaded	−18.50 ± 2.87	−28.11 ± 2.21	−21.82 ± 2.7
% EE		77.97 ± 1.38	73.52 ± 2.92	68.65 ± 3.35
% DL		27.46 ± 0.45	26.21 ± 0.30	23.89 ± 0.12
% Y		94.64 ± 2.48	93.51 ± 2.88	95.79 ± 1.47

[a] Ibrahim et al., 2013 (20). % EE the percentage encapsulation efficiency; % DL the percentage drug loading; %Y the percentage of NPs yield; PDI: polydispersity index

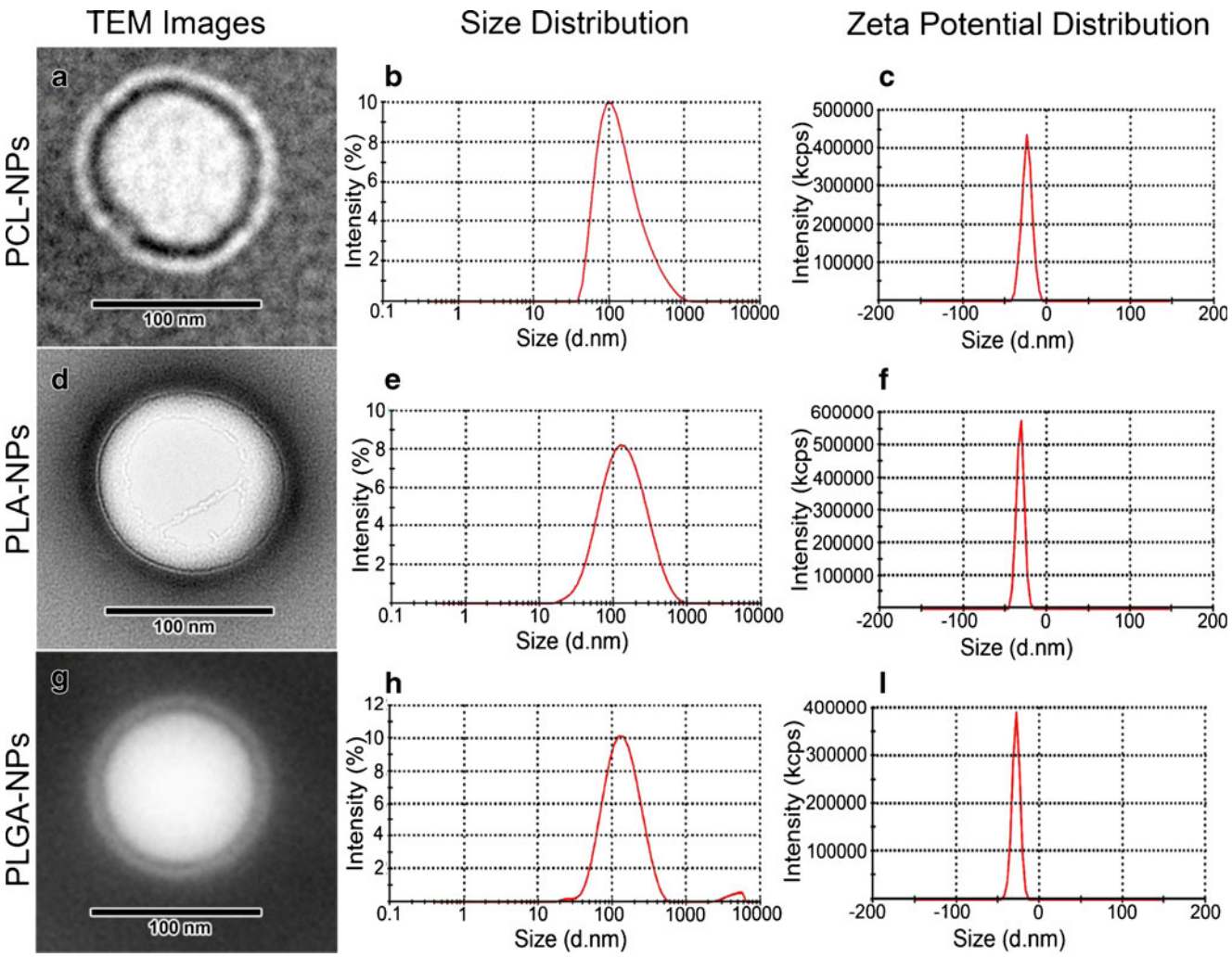


Fig. 1 TEM images, size and zeta potential distribution curves for brimonidine-loaded NPs. TEM image (**a**), size distribution curve (**b**) and zeta potential distribution curve (**c**) of PCL-NPs, TEM image (**d**), size distribution curve (**e**) and zeta potential distribution curve (**f**) of PLA-NPs, TEM image (**g**), size distribution curve (**h**) and zeta potential distribution curve (**i**) of PLGA-NPs.

102.65%. Figure 2 shows the rheological profiles of all ophthalmic formulations. The rheological profiles showed that eye drops had a Newtonian flow behavior, as at angular velocity 20 rpm or higher their viscosity did not change by increasing the shearing rate. In contrast, the *in situ* gelling system and preformed gel possessed a non-Newtonian pseudoplastic flow behavior, as their viscosity decreased by increasing the shearing rate over the entire range of the

Table III Characteristics of Ophthalmic Formulations Containing Brimonidine-Loaded NPs

Formulation		Evaluation (mean ± SD)		
		pH	Viscosity[a] (cP)	% Drug Content[b]
PCL-NP	Eye drops	7.82 ± 0.21	59.55 ± 1.61	99.12 ± 0.86
	In-situ gelling	7.75 ± 0.22	1172.85 ± 8.60	102.31 ± 2.96
	Preformed gel	7.73 ± 0.18	5638.57 ± 71.41	100.9 ± 1.51
PLA-NPs	Eye drops	7.81 ± 0.18	62.00 ± 1.05	98.79 ± 0.35
	In-situ gelling	7.74 ± 0.22	1212.40 ± 12.08	99.24 ± 1.53
	Preformed gel	7.75 ± 0.19	5380.48 ± 49.92	102.65 ± 0.81
PLGA-NPs	Eye drops	7.84 ± 0.21	60.25 ± 1.61	101.08 ± 0.49
	In-situ gelling	7.73 ± 0.23	1212.05 ± 5.39	100.31 ± 0.78
	Preformed gel	7.74 ± 0.22	5171.80 ± 65.74	98.78 ± 0.74

[a] Viscosity at 10 rpm and 35°C

[b] % Drug content (uniformity of drug content): The percentage of the available drug in the NP formulation with respect to the total amount of drug used when making NPs

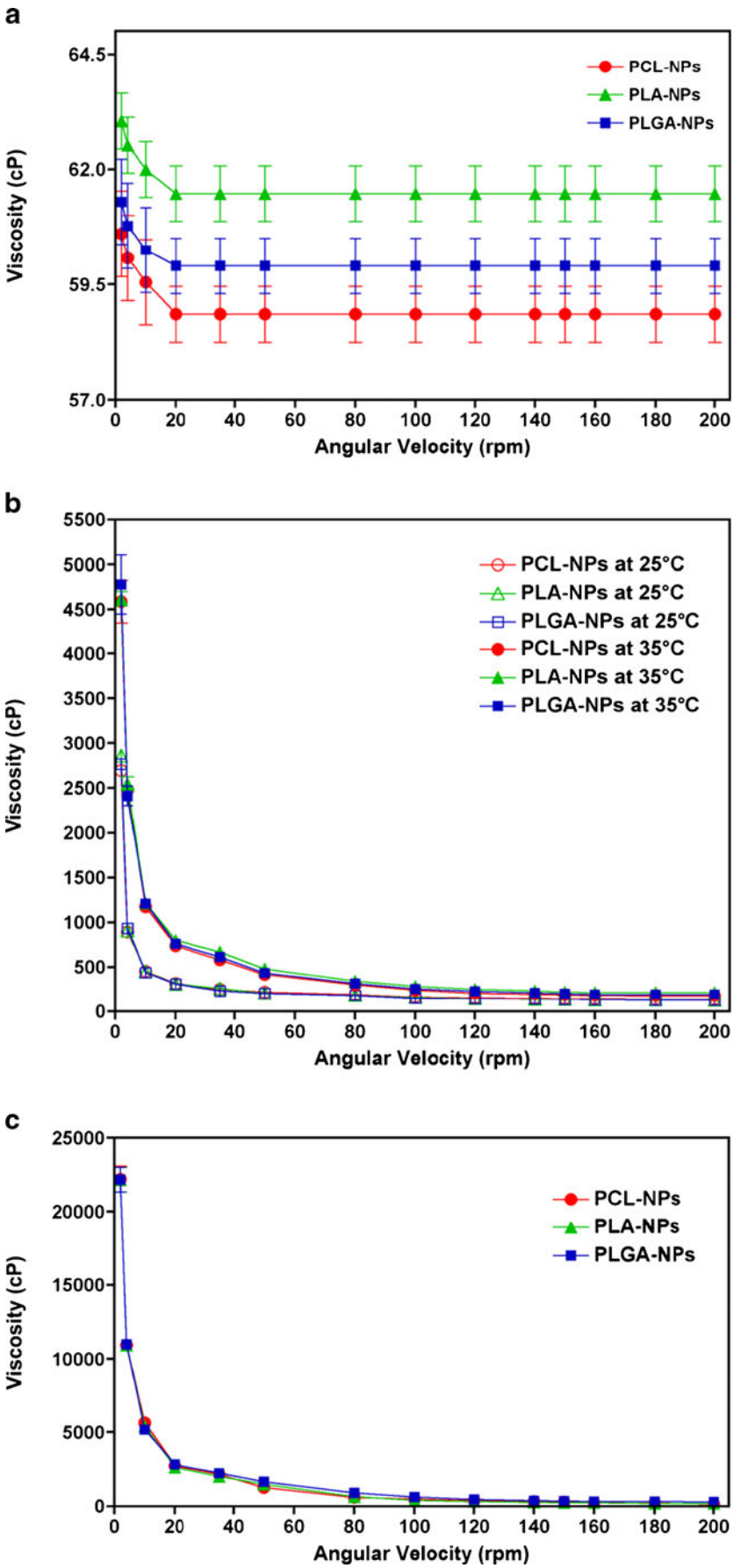


Fig. 2 Rheological profiles of brimonidine-loaded PCL-, PLA- or PLGA-NPs eye drops at 35°C (**a**), *in situ* gelling systems at 25 and 35°C (**b**), and preformed gels at 35°C (**c**).

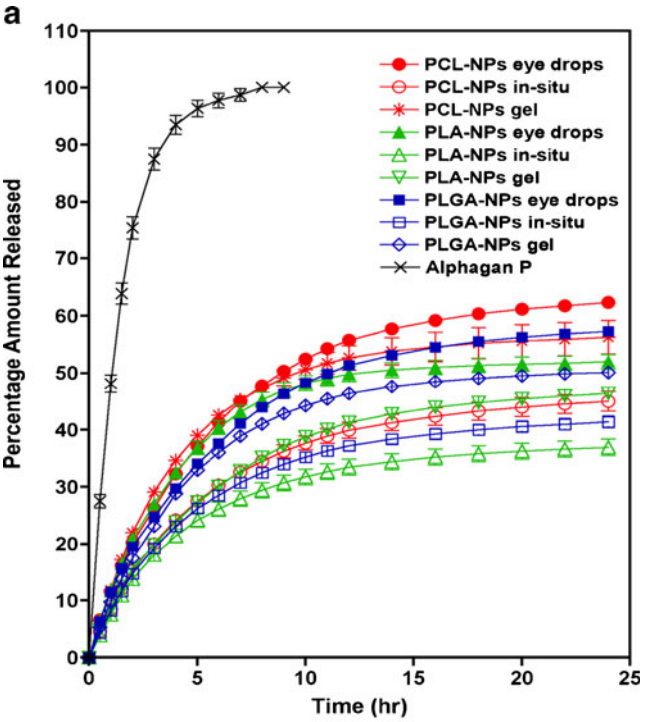
angular velocity. Overall, the behavior of our formulations, including the *in situ* gelling system, preformed gel and the eye drops below 20 rpm, are dependent on the angular velocity. Figure 2b shows the temperature-induced gelation of our *in situ* gelling system by increasing the temperature of the measurement from the non-physiological temperature (25°C) to the physiological temperature (35°C) (i.e. sol–gel transition).

Figure 3 shows the release profiles of our ophthalmic formulations and that of the control eye drops. Using the 25,000 Da molecular weight cut-off membranes, the cumulative amount of brimonidine released after 24 h from eye drops, gel and *in situ* gelling system respectively are: 62.3, 56.3 and 45.0% for PCL-NPs preparations; 57.3, 50.1 and 41.4% for PLGA-NPs preparations; and 52.0, 46.4 and 36.9% for PLA-NPs preparations (Fig. 3). In addition, the release of brimonidine from all preparations was significantly lower than that of Alphagan® P ($p<0.001$), which has a very rapid release pattern with 100% of the drug released within 8 h. The release of brimonidine from NPs made from the same polymer was significantly different when comparing dosage formulations. Eye drops had the greatest release, the preformed gel had an intermediate release, and the *in situ* gelling system had the least amount of brimonidine released during 24 h (Fig. 3a and b). Using one-way ANOVA followed by Tukey-Kramer multiple comparisons tests, there were no significant differences between the release profiles of all formulations using the three different molecular weight cut-off membranes. From this, we conclude that the release of brimonidine from our formulations is particle-controlled rather than membrane-controlled.

The results of our *in vitro* cell toxicity studies (Fig. 4) demonstrate that cell viability after exposure to our ophthalmic formulations ranged from 88.1% to 97.6%. There were statistically non-significant differences ($p<0.05$) between our formulations and the control preparation containing 0.1% brimonidine suspension. All formulations were significantly different ($p<0.001$) from the positive control, 1% of the cytotoxic agent Triton-X 100.

In Vivo Evaluation of the Ophthalmic Formulations

Upon instillation of the formulations into the eye of mice, all animals were calm and did not show any signs of ocular adverse effects or discomfort such as redness, irritation, burning, stinging or tearing for all the tested formulations. In contrast, administration of Alphagan® P eye drops resulted in immediate mild eye redness and lacrimation that lasted for 3–5 min after instillation of the eye drops. These transient side effects may be attributed to the high local drug concentration on the eye surface upon instillation of the eye drops (3).



b

PCL-NPs formulations	Eye drops	*In situ* gelling system
Eye drops	----	----
In situ gelling system	$P < 0.001$	----
Preformed gel	$P < 0.05$	$P < 0.001$

PLGA-NPs formulations	Eye drops	*In situ* gelling system
Eye drops	----	----
In situ gelling system	$P < 0.001$	----
Preformed gel	$P < 0.001$	$P < 0.001$

PLA-NPs formulations	Eye drops	*In situ* gelling system
Eye drops	----	----
In situ gelling system	$P < 0.001$	----
Preformed gel	$P < 0.01$	$P < 0.001$

Fig. 3 Brimonidine release profiles from ophthalmic formulations containing brimonidine-loaded PCL-, PLA- or PLGA-NPs and Alphagan® P drops (**a**), and statistical comparisons among final formulations of each nanoparticle (**b**).

The IOP profiles of both treated and control eyes after instillation of the ophthalmic formulations containing brimonidine-loaded PCL-, PLA- or PLGA-NPs or Alphagan® P eye drops into the right eyes of BXD29 (high IOP) and BXD96 (normal IOP) mice are shown in Fig. 5. The relevant pharmacodynamic data are listed in Tables IV and V. The results of IOP measurements of the control eyes (Fig. 5c and d) were statistically non-significantly different from the baseline IOP values ($p>0.05$). The IOP profiles of both treated high IOP and normal IOP mice (Fig. 5a and b) demonstrate that IOP continued to decrease for 5.2–7.2 h after application of our formulations after which time it began to slowly increase until it returned to baseline after 15.2–23.2 h. In contrast, Alphagan P® gave its maximum IOP decrease after 2–2.2 h and its IOP lowering effect was terminated by 7–7.4 h.

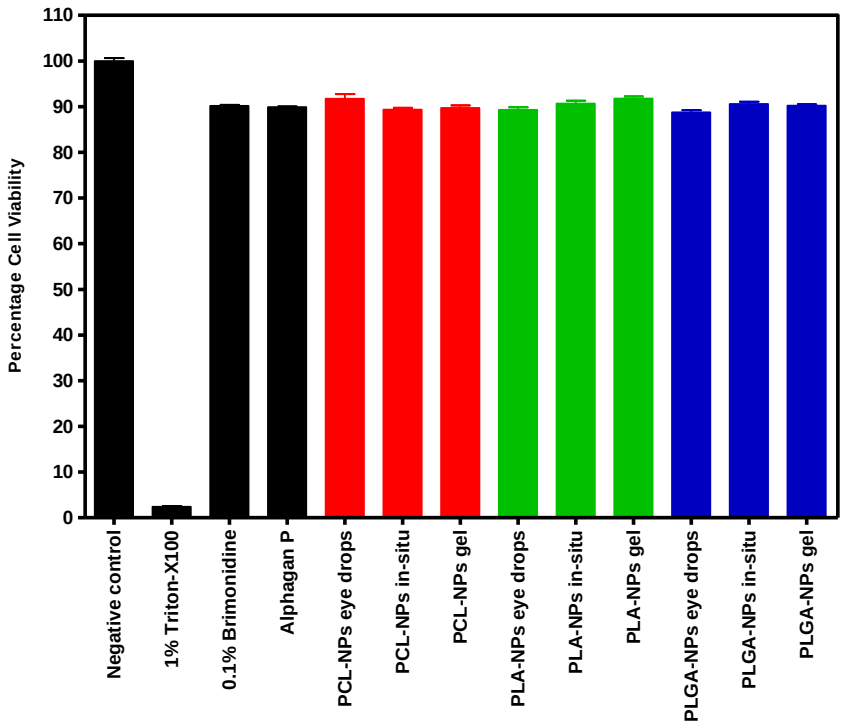


Fig. 4 Cytotoxicity histograms of ophthalmic formulations containing brimonidine-loaded PCL-, PLA- or PLGA-NPs.

Tables IV and V list the various phamacodynamic parameters after administration of ophthalmic formulations. There was no significant difference between the I_{max} values of our formulations and that of Alphagan® P for both high and normal IOP mice; all formulations decreased the IOP to the same limit ($p>0.05$). In contrast, there was a significant difference between the I_{max} values of PCL-NPs eye drops and both PLA- and PLGA-NPs eye drops for BXD96 mice ($p<0.05$). Comparison of the T_{max} values of the tested formulations and that of Alphagan® P demonstrate that our ophthalmic formulations possessed significantly delayed T_{max} values for both BXD mice strains. In contrast, there was no significant difference between the T_{max} values of all of our formulations ($p>0.05$). Regarding the T_{end} values, our formulations significantly extended the duration of the IOP lowering effect of brimonidine in both mice strains ($p<0.001$ for BXD29, and $p<0.01$ for BXD96 mice) compared to Alphagan® P. There were no significant differences among the T_{end} values of our formulations, except between PLGA-NPs gel and eye drops when used in BXD29 (high IOP) mice ($p<0.05$). Also our data show that both the AUC_{total} and AUC_{rel} of our formulations were highly significantly different from those of Alphagan® P for both mice strains ($p<0.01$) for all eye drops and ($p<0.001$) for gels and *in situ* gelling system. Furthermore, there were no significant differences between our formulations other than between PCL-NPs gel and PLA-NPs eye drops at ($p<0.05$). Also our data demonstrate that the AUC_{total} and AUC_{rel} of PCL-NP formulations were higher than those of PLGA-NP formulations, which were higher than those of PLA-NPs formulations. Upon comparing the AUC_{total} and AUC_{rel} of different dosage forms it was found preformed gel > *in situ* gelling system > eye drops. Figure 5 illustrates that the IOP of the treated eyes was decreased after application of our formulations, while that of control eyes did not change, which indicates that the drug did not crossed over from the treated eye to the control one (compare Fig. 5a to c and b to d).

To determine the release mechanism kinetic model that best describes the pattern of drug release from our formulations, our *in vitro* release data were analyzed according to zero-order, first-order and Higuchi diffusion models (Table VI). The preference for a particular model was based on the correlation coefficient, with the highest coefficient indicating the release mechanism (25). We then used the Korsmeyer-Peppas semi-empirical model for further in depth analysis (26), where the value of the release exponent depends on the release mechanism and thus could be used to characterize it (27). Results of our kinetic analyses showed that brimonidine release from all formulations as well as Alphagan® P eye drops followed a Higuchi model. This suggests that drug release is governed by a diffusion release mechanism. Further analysis of the release data using the Korsmeyer-Peppas equation showed that the release exponents (n) for all formulation ranged between 0.45 and 0.89, indicating that they exhibit a non-Fickian or anomalous diffusion, meaning that drug release from our formulations did not occur by a pure diffusion mechanism, but is rather a mixture of both diffusion from NPs and erosion of the polymers. In contrast, the value of (n) for Alphagan® P eye drops is 0.4511, which suggests a Fickian diffusion mechanism whereby the release occurs by a pure diffusion mechanism (27).

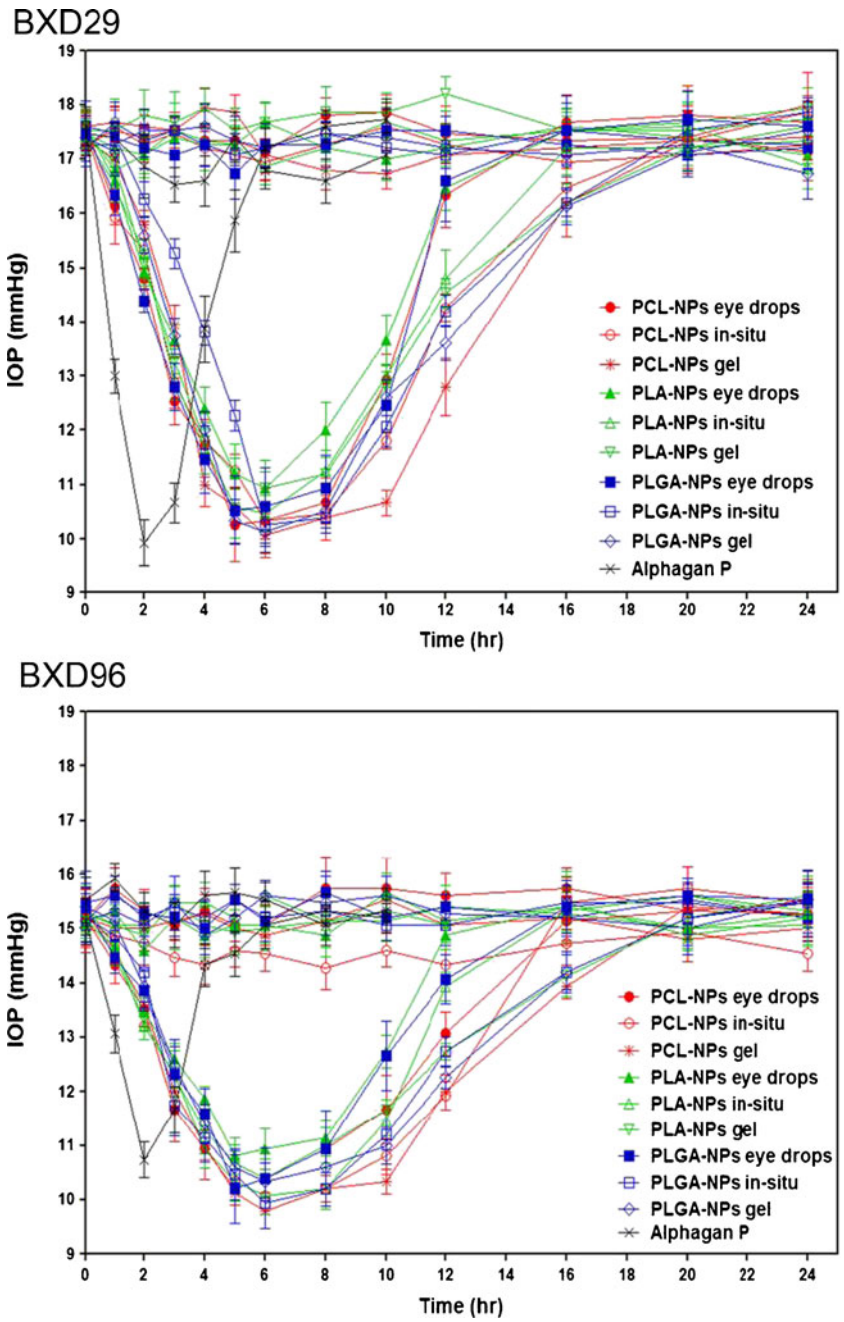


Fig. 5 24 h IOP profiles after a single application of the ophthalmic formulations containing brimonidine-loaded PCL-NPs, PLA-NPs or PLGA-NPs or Alphagan® P drops to the right eye of BXD mice. Left eyes did not receive any drops. The IOP reduction was statistically significant.

DISCUSSION

In this investigation, we sought to develop an improved topical formulation of brimonidine for the treatment of elevated IOP. To accomplish this goal, we synthesized NPs from three synthetic polymers and incorporated each into three topical formulations. All formulations provided extended release and exhibited improved IOP-lowering capabilities compared to Alphagan® P.

There are several reasons for the increased efficacy of our formulations including the use of the free-base form of the drug, the encapsulation of brimonidine in NPs to allow for extended release, and the inclusion of the NPs in dosage forms that discourage rapid elimination from the surface of the eye. Other features of our optimal formulations include a neutral pH, a non-toxic nature, and the pseudoplastic flow behavior of our gel-based topical formulations.

We chose to use brimonidine free-base rather than brimonidine tartrate salt to facilitate absorption into the eye. The inclusion of free-base is advantageous, as it is non-ionized and is more readily absorbed through the cornea and conjunctiva by passive diffusion upon release from the NPs.

Table IV Pharmacodynamic Parameters After Administration of Ophthalmic Formulations Containing Brimonidine-Loaded PCL-NPs, PLA-NPs, PLGA-NPs or Alphagan® P Eye Drops to BXD29 (High IOP) Mice

Parameters formulations		Pharmacodynamic parameters (mean ± SEM)				
		I_{max} (mmHg)	T_{max} (h)	T_{end} (h)	AUC_{total} (mmHg.h)	AUC_{rel} (mmHg.h)
	Control eye drops	9.87 ± 0.71	2.2 ± 0.2	7.0 ± 0.89	25.94 ± 0.91	1.0 ± 0.04
PCL-NPs	Eye drops	9.2 ± 0.71	6.0 ± 0.55	16.0 ± 2.19	63.61 ± 8.84	2.45 ± 0.34
	In situ gelling	10.07 ± 0.32	6.4 ± 0.4	20.8 ± 1.5	70.89 ± 5.83	2.73 ± 0.23
	Preformed gel	9.6 ± 0.62	6.6 ± 1.4	21.6 ± 0.98	74.61 ± 3.06	2.88 ± 0.12
PLA-NPs	Eye drops	10.87 ± 0.67	5.6 ± 0.25	18.4 ± 2.4	53.99 ± 5.73	2.08 ± 0.22
	In situ gelling	9.8 ± 0.62	5.4 ± 0.25	20.0 ± 1.79	60.26 ± 3.82	2.32 ± 0.15
	Preformed gel	10.87 ± 0.44	5.6 ± 0.25	21.6 ± 0.98	64.92 ± 4.64	2.5 ± 0.18
PLGA-NPs	Eye drops	9.4 ± 0.76	6.0 ± 0.55	15.2 ± 2.33	58.59 ± 11.3	2.26 ± 0.44
	In situ gelling	10.07 ± 0.25	7.2 ± 0.49	22.4 ± 0.98	66.06 ± 1.94	2.55 ± 0.08
	Preformed gel	10.13 ± 0.58	5.4 ± 0.25	23.2 ± 0.8	70.19 ± 3.44	2.71 ± 0.13
Statistical parameters	Overall *p* value	0.5583	0.0001	0.0001	0.0001	0.0001
	F value	0.8709	5.536	9.201	5.635	5.636
	Degree of freedom	9	9	9	9	9

I_{max} Maximum decrease in IOP, T_{max} Time required to reach maximum decrease in IOP, T_{end} Time required for IOP to return again to its baseline (i.e. end of drug effect), AUC_{total} Total area under IOP *versus* time curve, AUC_{rel} Relative area under IOP *versus* time curve which can be estimated from the quotient of the values of AUC_{total} of the ophthalmic formulations and that of the control preparation. Data are presented as mean ± SEM and $n = 5$

In contrast, Alphagan® P contains brimonidine tartrate salt—the ionized form—the absorption of which depends mainly on the pH of the eye surface (28). The free-base form of brimonidine also allows for a greater encapsulation efficiency in NPs. Because the salt has high water solubility (2), it would be lost in the aqueous phase during the emulsification step, which would yield lower encapsulation efficiency. Brimonidine base is poorly water-soluble, therefore it was preferentially partitioned in the organic phase of the emulsion and only a small amount of drug was lost in the aqueous phase. Another reason for the high encapsulation efficiency in our study is the small particle size we achieved due to the

Table V Pharmacodynamic Parameters After Administration of Ophthalmic Formulations Containing Brimonidine-Loaded PCL-NPs, PLA-NPs, PLGA-NPs or Alphagan® P Eye Drops to BXD96 (Normal IOP) Mice

Parameters formulations		Pharmacodynamic parameters (mean ± SEM)				
		I_{max} (mmHg)	T_{max} (h)	T_{end} (h)	AUC_{total} (mmHg.h)	AUC_{rel} (mmHg.h)
	Control eye drops	10.2 ± 0.39	2.4 ± 0.25	7.4 ± 0.87	12.73 ± 2.29	1.0 ± 0.18
PCL-NPs	Eye drops	8.53 ± 0.44	5.4 ± 0.81	16.0 ± 2.83	44.43 ± 4.4	3.49 ± 0.35
	In situ gelling	9.2 ± 0.23	5.8 ± 0.66	16.8 ± 0.8	52.35 ± 6.59	4.11 ± 0.52
	Preformed gel	9.8 ± 0.34	5.4 ± 0.25	22.4 ± 0.98	58.96 ± 4.14	4.63 ± 0.33
PLA-NPs	Eye drops	10.73 ± 0.49	5.2 ± 0.2	15.2 ± 0.8	33.17 ± 3.4	2.61 ± 0.27
	In situ gelling	9.2 ± 0.23	5.8 ± 0.66	16.0 ± 2.19	42.03 ± 5.53	3.30 ± 0.44
	Preformed gel	10.27 ± 0.41	5.4 ± 0.4	20.8 ± 1.5	49.98 ± 2.72	3.93 ± 0.21
PLGA-NPs	Eye drops	8.53 ± 0.44	5.4 ± 0.81	18.8 ± 3.2	41.89 ± 8.2	3.29 ± 0.64
	In situ gelling	9.8 ± 0.53	6.4 ± 0.4	20.0 ± 1.79	51.97 ± 6.19	4.08 ± 0.49
	Preformed gel	10.33 ± 0.55	5.4 ± 0.25	22.4 ± 0.98	54.71 ± 5.06	4.3 ± 0.4
Statistical parameters	Overall *p* value	0.004	0.0008	0.0001	0.0001	0.0001
	F value	3.332	4.122	6.134	6.735	6.735
	Degree of freedom	9	9	9	9	9

I_{max} Maximum decrease in IOP, T_{max} Time required to reach maximum decrease in IOP, T_{end} Time required for IOP to return again to its baseline (i.e. end of drug effect), AUC_{total} Total area under IOP *versus* time curve, AUC_{rel} Relative area under IOP *versus* time curve which can be estimated from the quotient of the values of AUC_{total} of the ophthalmic formulations and that of the control preparation. Data are presented as mean ± SEM and $n = 5$

Table VI *In Vitro* Release Kinetics of Brimonidine

Formula		Correlation coefficient (r^2)			Release mechanism	Korsmeyer-Peppas		Drug transport mechanism
		Zero	First	Higuchi		n	r^2	
	Control	0.7025	0.6002	0.9066	Diffusion	0.4511	0.9072	Fickian
PCL-NPs	Eye drops	0.7906	0.6097	0.9455	Diffusion	0.5915	0.9537	Non-Fickian
	In-situ gelling	0.7603	0.5841	0.9155	Diffusion	0.5893	0.9333	Non-Fickian
	Performed gel	0.6767	0.5213	0.9096	Diffusion	0.5565	0.9154	Non-Fickian
PLA-NPs	Eye drops	0.6696	0.5173	0.9035	Diffusion	0.5436	0.9240	Non-Fickian
	In-situ gellig	0.7111	0.5448	0.9078	Diffusion	0.5737	0.9202	Non-Fickian
	Performed gel	0.7927	0.6087	0.9447	Diffusion	0.5866	0.9488	Non-Fickian
PLGA-NPs	Eye drops	0.7913	0.6141	0.9461	Diffusion	0.5731	0.9543	Non-Fickian
	In-situ gellig	0.7542	0.5744	0.9203	Diffusion	0.5748	0.9323	Non-Fickian
	Performed gel	0.7338	0.5609	0.9093	Diffusion	0.5862	0.9283	Non-Fickian

cooperation of all formulation factors including the use of acetone. Upon organic phase injection into the aqueous phase during NP preparation, acetone, a highly diffusible solvent, is removed rapidly leaving the poorly water-soluble drug within NPs away from the external aqueous phase. These results are in accord with those obtained by Kim *et al.* (29) who reported an improvement in the encapsulation efficiency of celecoxib in PLGA-NPs when using acetone as a solvent compared to other solvents such as dimethylformamide, dimethylsulfoxide, 1,4-dioxane and dimethylacetamide.

In our study, we found that the encapsulation efficiency of brimonidine was ranked in the following order: PCL-NPs > PLA-NPs > PLGA-NPs. This may be due to the difference in polymer hydrophobicity, as it have been established that the hydrophobicity of the polymers is greatest for PCL and least for PLGA, with PLA having an intermediate hydrophobicity (17). An increase in the hydrophobic nature of the polymer leads to an increased solid-state solubility of the hydrophobic drug in the polymer resulting in enhanced encapsulation efficiency (30).

Dosage form vehicles also play an important role in improving the efficacy of treatment by increasing the contact time of the drug with the surface of the eye, thus improving its bioavailability. Specifically, the eye drops and the *in situ* gelling system contained HPMC while the preformed gel consisted of MC, both of which are bioadhesive cellulose derivatives that function to prolong contact time on the eye (31). Among our formulations, the ranking of bioavailability—preformed gel > *in situ* gelling system > eye drops— may be due to the differences in their viscosity profiles. The Newtonian flow behavior of the eye drops is likely due to its HPMC content, as this material has been shown to exhibit Newtonian flow behavior when used alone without other additives either in physiological or non-physiological conditions (32). In contrast, the rheological profiles of the *in situ* gelling system and preformed gel are characterized by non-Newtonian pseudoplastic (i.e. shear thinning) flow behaviors that are due to the presence of poloxamer 188 (33) and MC (34), respectively. Pseudoplastic flow behavior is preferred for topical ophthalmic preparations because it matches the huge variations in shear rate that are experienced on the ocular surface; during the inter-blinking rest periods, the ocular shear rate is very low ($0.03\ s^{-1}$), yet it is very high during blinking ($4{,}250–28{,}500\ s^{-1}$) (12). The pseudoplastic characteristics of our *in situ* gelling system and preformed gel allow for higher viscosities during inter-blinking periods to prevent their drainage from the eye, yet during blinking, they have very low viscosities that would not cause patient discomfort.

In addition to matching the viscosity requirements of the corneal surface, our formulations exhibit a sustained drug release rate that is free of any burst release that may be toxic. The burst release is a characteristic of drugs released from monolithic NPs systems in which drug molecules are located at or near the surface of the NP. In our study, the absence of a burst release may be due to two factors. The first is that the NPs are not suspended directly in dissolution medium but rather are dispersed in dosage form vehicles (i.e. eye drops, *in situ* gelling system or gel). Because of this, once the drug is released from the NPs, it is further dispersed in vehicle prior to release in the dissolution medium (18). The second factor that may inhibit the burst release is the presence of a coat around the NPs that was confirmed by TEM data. This coat may prevent drug from adhering to the surface of NPs during synthesis and thus no drug was available for burst effect. In addition, this coat shielded the NP surface which further mitigated the possibility of a rapid release of drug upon exposure to the release medium (18). In the literature there are many studies that have induced a coat around NPs using various materials to decrease or prevent the burst release (35–37). Our method of synthesizing NPs generated a coating during production, thereby minimizing the steps required to optimize the release kinetics.

Our data demonstrate that within any one formulation, the rate of brimonidine release was ranked PCL-NPs > PLGA-NPs > PLA-NPs. This may be due to difference in NP sizes because smaller particle sizes have higher surface areas available for drug release which would translate into higher the release rates (38). Another factor that may affected brimonidine release rates is the difference in molecular weights of the polymers used for NPs synthesis because higher molecular weight polymers exhibit slower polymer degradation rates, which would cause a slower release rate of the drug from NPs (30).

Our formulations are highly biocompatible, which adds to their benefit. The pH of eye tears is 7.4 and due to the eye natural buffering capacity, it can tolerate ophthalmic formulations within a wide pH range (3.5 to 8.5) and rapidly restore back to its normal pH value. The typical ophthalmic dose is normally 1 or 2 drops, which is a small volume that can be easily accommodated by the eye in terms of volume and buffering capability (24). The pH of all of our formulations was 7.7–7.8, which can be easily tolerated by the eye without any irritation or discomfort. In addition, our formulations were non-toxic, even when a maximal volume was tested. In our cell toxicity studies, the volume of each formulation was 65 μl; the typical volume of topical eye drops range between 33.8 and 63.4 μl (39). Our data show that all formulations as well as plain dosage form vehicles, plain NPs, 0.1% brimonidine suspension, and Alphagan® P eye drops were non-toxic. These highly favorable results may be due to the fact that all polymers we selected to use in our formulations—both dosage (40) and NPs (16,17)— possessed an excellent biocompatibility.

CONCLUSION

In conclusion, this paper presents the preparation and evaluation of new topical ophthalmic sustained release dosage forms containing brimonidine-loaded PCL-, PLA- or PLGA-NPs prepared by a spontaneous emulsification solvent diffusion method. The optimized NP formulations had desirable particle sizes, zeta potential and surface morphology. The formulations possessed pH and viscosity values that are compatible with the eye and have uniform drug contents that comply with the USP official requirement. The release of brimonidine from all ophthalmic formulations showed a sustained release free from any burst effect with the formulations following a Higuchi non-fickian diffusion mechanism. All formulations were non-toxic. Moreover, a single topical dose maintained a reduced IOP for greater than 16 h. Collectively all data effectively demonstrate that we have developed optimized formulations for sustained delivery of brimonidine for management of glaucoma. A once daily formulation should meet with better patient compliance and more effective control of IOP.

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