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Once Daily Pregabalin Eye Drops for Management of Glaucoma

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Abstract

Elevated intraocular pressure (IOP) is the most significant risk factor contributing to visual field loss in glaucoma. Unfortunately, the deficiencies associated with current therapies have resulted in reduced efficacy, several daily dosings, and poor patient compliance. Previously, we identified the calcium voltage-gated channel auxiliary subunit alpha2delta 1 gene (*Cacna2d1*) as a modulator of IOP and demonstrated that pregabalin, a drug with high affinity and selectivity for CACNA2D1, lowered IOP in a dose-dependent manner. Unfortunately, IOP returned to baseline at 6 h after dosing. In the current study, we develop a once daily topical pregabalin-loaded multiple water-in-oil-in-water microemulsion formulation to improve drug efficacy. We characterize our formulations using multiple *in vitro* and *in vivo* evaluations. Our lead formulation provides continuous release of pregabalin for up to 24 h. Because of its miniscule droplet size (<20 nm), our microemulsion has a transparent appearance and should not blur vision. It is also stable at one month of storage at temperatures ranging from 5 to 40 °C. Our formulation is nontoxic, as illustrated by a cell toxicity study and slit-lamp biomicroscopic exams. CACNA2D1 is highly expressed in both the ciliary body and the trabecular meshwork, where it functions to modulate IOP. A single drop of our lead pregabalin formulation reduces IOP by greater than 40%, which does not return to baseline until >30 h post-application. Although there were no significant differences in the amplitude of IOP reduction between the formulations we tested, a significant difference was clearly observed in their duration of action. Our multilayered microemulsion is a promising carrier that sustains the release and prolongs the duration of action of pregabalin, a proposed glaucoma therapeutic.

Graphical Abstract

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The authors declare the following competing financial interest(s): Drs. Jablonski and Ibrahim have a patent pending on the microemulsion presented in this paper.

Keywords

glaucoma; microemulsion; corneal permeability; immunohistochemistry; cytotoxicity; XRD; stability

Glaucoma is a group of ophthalmic diseases characterized by progressive visual field loss due to damage to the optic nerve. Persistent or repeated elevation of intraocular pressure (IOP) is a primary risk factor. Primary open angle glaucoma (POAG) accounts for 90% of glaucoma cases worldwide, and it is a leading cause of irreversible blindness.¹ The balance of aqueous humor (AH) production by the ciliary body (CB) and its drainage through conventional trabecular meshwork (TM) pathway, and to a lesser degree by the nonconventional uveoscleral pathway, lead to a steady-state IOP. However, in glaucoma, a high IOP is generated by an imbalance between the AH inflow and its outflow. Fortunately, IOP can be medically controlled; thus, IOP reduction is the first-line therapeutic option in glaucoma.² Therefore, the mode of action of current medications includes a decrease of AH production or increase of AH outflow by the TM or uveoscleral pathway or both.^{3,4}

Through our systems genetics investigations, we identified *Cacna2d1*, a subunit of the L-type calcium channel, as a gene modulator of IOP. Our previous study demonstrated that CACNA2D1 is localized to the CB (area of AH production) and TM (area of conventional AH drainage) in both mouse and human eyes leading us to predict that pregabalin (PRG), a gabapentinoid drug with high affinity and high selectivity for CACNA2D1, would lower IOP by decreasing the production or increasing the drainage of aqueous humor from the eye.⁵ We demonstrated that topical installation of PRG eye drops reduced IOP in a dose-dependent manner. However, the IOP-lowering effect was limited to 6 h.⁵ To improve the efficacy of PRG, we sought to engineer an extended release formulation for once daily use.

Despite the availability of several FDA-approved drugs (*e.g.*, prostaglandin analogs, carbonic anhydrase inhibitors, and beta-blockers) to lower IOP, these agents are not a cure for POAG. Moreover, they are not universally effective in reducing IOP, and they are laden with multiple side effects.^{6–8} Accordingly, patient compliance is low and visual deterioration is frequent, resulting in severe impairment and blindness in millions of people around the

world. Although there are two compounds have recently received FDA approval for treatment of POAG, Rhopressa (suppressor of rho kinase enzymes) and Vyzulta (dual mechanism prostaglandin analog and producer of nitric oxide), as once daily eye drops, unfortunately, both medications are associated with serious side effects, which include conjunctival hyperemia (53% of patients), instillation site pain (~20% of patients), conjunctival hemorrhage (~20% of patients), cornea verticillata (~20% of patients) with Rhopressa,⁹ and changes in iris pigmentation with Vyzulta.¹⁰ Therefore, the advancement of an effective drug with high affinity and selectivity to a specific target protein in ocular tissues that modulate IOP while minimizing the risk of side effects is still an urgent unmet medical need. Because CACNA2D1 is localized to tissues involved in AH production and outflow, it is plausible that PRG may function at both sites. A dual mechanism of action would significantly enhance drug efficacy and would generate a promising class of glaucoma medications.

Eye drops are the most commonly used and acceptable topical ophthalmic dosage form all over the world.^{11,12} When the active ingredient is a water-soluble drug, this dosage form usually consists of a simple drug solution. The prevalence of this dosage form is likely due to the ease of manufacturing and the ease of application to the eye. Unfortunately, this dosage form suffers from several drawbacks (*e.g.*, rapid drainage of drug from the eye surface either to the outside of the eye or to the systemic circulation through nasolacrimal duct, and the very short corneal contact time) that may result in absorption of only 5% of the administered dose into ocular tissues.^{13,14} Several daily dosings may overcome these shortcomings, yet they usually result in exaggeration of systemic side effects, lower patient satisfaction, and its associated compliance along with an increase in ocular surface problems that may collectively cause the patient to discontinue drug use. Unfortunately, reduced compliance usually leads to an increase in visual field loss.

Several formulations strategies have been used to overcome the limitations associated with aqueous solution eye drops, among them are viscosity-inducing agents,^{15–17} penetration enhancers,¹⁸ bioadhesive polymers,¹⁹ ocular implants,^{13,20,21} contact lenses,^{22,23} ocular inserts,²⁴ ocular injections,^{25,26} and colloidal drug delivery systems (*e.g.*, liposomes, nanosuspensions, microparticles, nanoparticles,²⁷ nanoemulsions, and microemulsions²⁸). While all the listed systems may sustain drug release or prolong corneal contact time, microemulsions offer several advantages that make them superior to other colloidal drug delivery systems.

A microemulsion (ME) is defined as a single-phase, isotropic, thermodynamically stable system with a droplet size in the submicron range (10–100 nm diameter).²⁹ MEs are stabilized by a surfactant usually in combination with a cosurfactant, which may be an amine, a short chain alcohol, or another weakly amphiphilic molecule.^{30,31} They have multiple advantages including thermodynamic stability, optical clarity, ease of preparation and scaling up, few synthesis steps, small energy requirement during preparation, 100% encapsulation efficiency, and compatibility with both water-soluble and water-insoluble drugs.

Because of the excellent characteristics of MEs, multiple studies demonstrated their efficient application in improving the solubility of poorly water-soluble drugs. Despite their success in increasing the absorption and bioavailability of hydrophobic molecules,^{32–34} MEs have not yet been developed to improve the efficacy of highly water-soluble drugs. Our goal in this investigation was to develop a ME to slow the release and control the diffusion of PRG, a BCS class I molecule that represents a promising class of glaucoma therapeutics. By optimizing the extended release characteristics of a multiple water-in-oil-in-water (W/O/W) ME with bioadhesive properties, we have generated and validated a once daily drop that maintains a reduced IOP for greater than 30 h.

RESULTS AND DISCUSSION

We have previously demonstrated that PRG could potentially serve as a promising drug candidate for management of POAG.⁵ PRG, *aka* (S)-3-aminomethyl-5-methylhexanoic acid, has been clinically effective in treating epileptic seizures or neuropathic pain disorders such as fibromyalgia and diabetic peripheral neuropathy.^{35,36} It is currently marketed by Pfizer (NY, USA) as Lyrica and has become a lead molecule due to its hydrophilicity, rapid absorption after oral administration, and high bioavailability (~90%).³⁷ Unfortunately, its hydrophilicity may hinder its topical ophthalmic use because like other hydrophilic molecules, it will drain rapidly from the eye surface, which leads to low corneal residence time and short duration of action. In the current study, we endeavored to improve the corneal contact time and the duration of action of PRG by incorporating it in specially engineered bioadhesive W/O/W ME eye drops. Our main goal in this study was to prepare an efficient IOP-lowering, once daily topical ophthalmic formulation with expected better patient compliance.

Ternary and Pseudo-Ternary Phase Diagrams and Preparation of Multiple W/O/W ME Eye Drops.

The solubility of PRG in different aqueous media was analyzed to screen components for the internal aqueous phase of our W/O/W multiple microemulsion. Among the three aqueous media tested (Milli-Q water, 0.01N HCL, and 0.01N NaOH), PRG solubility was higher in 0.01N HCL (41.48 ± 0.17 mg/mL) than in both Milli-Q water (37.7 ± 0.08 mg/mL) and 0.01N NaOH (39.28 ± 0.15 mg/mL). However, Milli-Q water was chosen as the internal aqueous phase for further studies in the ternary and pseudo-ternary phase diagrams due to an observed physical incompatibility with both 0.01N HCL and 0.01N NaOH. In addition, PRG solubility was also determined in Labrafac lipophile WL1349 oil (Labrafac oil) and the surfactant mixture of the water-in-oil (W/O) ME (Capryol 90 and soybean lecithin, 1:1 mixture) to test their ability in controlling the drug release later from the internal aqueous phase. Interestingly, the PRG concentration in both Labrafac oil and the surfactant mixture was under the detection limit of our HPLC method, suggesting that both the oil and the surfactant mixture could slow the drug diffusion through these layers of the multiple W/O/W ME due to the insolubility of PRG in these components.

In the preparation of a stable ME system, the hydrophilic–lipophilic balance (HLB) value of the surfactant used to generate the W/O ME and O/W ME should fall in the range of 3–6 and

8–18, respectively.³⁸ In our current work, we followed this rule during the formulation of our ME. Specifically, during the preparation of the primary W/O ME, we selected surfactants with low HLB value (< 5) such as Capryol 90, lecithin, and transcutol P. In addition, during the preparation of the final W/O/W ME, all the used surfactants, such as Labrasol, tween 80, poloxamer 188, brij 97, and Cremophor EL, possessed high HLB values (> 12).

Figure 1 illustrates the constructed ternary and pseudo-ternary phase diagrams with the W/O ME illustrated by the shaded regions. These regions represent all possible combinations of the three microemulsion components, aqueous phase, oil phase, and surfactant or surfactant mixture, that were capable of forming a transparent primary W/O ME. By selecting any point in that region, we identify the percentage of the three components that are easily mixed to form a W/O ME. Figure 1B was chosen to select one point to prepare our W/O ME because it had the largest shaded area, which gave us a larger number of combinations for selection. The selected W/O ME point consisted of 20% Milli-Q water +30% Labrafac oil +50% surfactant mixture (Capryol 90 and soybean lecithin, 1:1). Two important criteria should be available in the point to be selected: the first is its ability to form a stable primary W/O ME, and the second criterion is the ability to incorporate the highest possible percentage of Milli-Q water as an internal phase to dissolve as much as possible of the required drug dose (0.6% PRG; this PRG dose is the minimum concentration that gives the maximum reduction in IOP, data not shown).

Examination of the structure of the used chemicals to produce the selected pseudo-ternary phase diagram provides an understanding of the larger ME area that leads to the preference of the ternary and pseudo-ternary phase diagrams. Capryol 90 is a propylene glycol monoester of caprylic acid (C₈). Because it is a medium hydrocarbon chain, it can readily insert between the two long hydrocarbon chains of lecithin to form a homogeneous emulsifying film at the interface between Labrafac oil and Milli-Q water.³⁹ Our oil phase (Labrafac oil) is a medium chain triglyceride ester of caprylic (C₈) and capric (C₁₀) acids, which possesses nearly the same carbon chain length of Capryol 90. We predict that this supports more homogeneous mixing between our ingredients and better emulsification behavior. Labrafac oil, a medium chain triglyceride ester, has a higher solvent capacity and is less susceptible to oxidation than long chain triglycerides.⁴⁰ Also, being an ester of saturated medium chain acids (C₈ and C₁₀), our oil phase possessed higher shelf stability compared to those containing unsaturated centers because unsaturated centers are more likely to be attacked by free radicals or any oxidizing species during its storage.⁴¹

In addition to being physically compatible with Capryol 90, lecithin was selected as a surfactant in our ME because it has the advantage of being a safe, biocompatible, nontoxic, naturally occurring, and well-tolerated surfactant.^{31,39} Furthermore, it is, in many respects, regarded as an ideal biological surfactant because of its biodegradation and its phospholipid composition that resemble the structure of cell membranes.⁴² Despite of all these advantages, lecithin possesses undesirable properties that may hinder its use as a surfactant for preparation of ME. Specifically, it has two opposite characteristics in that it has strong hydrophobicity due to the presence of its two long hydrocarbon chains, while at the same time it has strong lipophilicity, due to the presence of hydrated zwitterionic polar groups,

and a strong tendency to form lamellar liquid crystalline phase. This problem could be overcome by combining it with other cosurfactants to decrease its rigidity.³⁹ Capryol 90 provides our ME with this advantage and decreases the rigidity of lecithin by being incorporated into the interfacial film and allowing the formation of ME.

Among the used surfactants and cosurfactants, a surfactant mixture of 7.4% Labrasol and 7.4% Cremophor EL in the presence of 22.2% propylene glycol as a cosurfactant was the most efficient system to produce our final multiple W/O/W ME (Table 1). This combination of surfactants and cosurfactant helps to incorporate the maximum percentage of the primary W/O ME (26% w/w) during the final emulsification process to prepare our final multiple W/O/W ME. Regarding the safety of our ME system, several studies previously conducted and demonstrated the safety of our chemicals at the concentrations used in our W/O/W ME. Fialho *et al.* used Cremophor EL at a concentration of 15% in their ophthalmic ME formulation as a safe administration of dexamethasone to the eye.⁴³ Also, they stated that according to the information provided by the manufacturer, Cremophor EL at a concentration up to 30% does not have any irritant effect on the eye.^{43,44} Furthermore, a solution of propylene glycol is safe and causes no irritations to the rabbit eye at a concentration up to 50%, whereas undiluted propylene glycol only associated with a weak conjunctival redness.^{42,45,46} Similarly, Liu *et al.* reported that Labrasol at a concentration of 5% produced a slight ocular irritation when used as ophthalmic penetration enhancer for their drug (baicalin).⁴⁷ Despite using Labrasol as a surfactant in our ME at a concentration above the limit that was previously reported, our results demonstrate that it does not produce any ocular irritation at all. The excellent safety of our ME in the eye may be due to the demulcent effect of propylene glycol that is capable of masking the potential irritation produced by Labrasol at the used concentration.⁴⁸

pH, Average Droplet Size, Polydispersity Index (PDI), and Zeta Potential (ZP).

Our developed PRG-loaded chitosan, sodium alginate, and Carbopol 981 ME eye drops have pH values of 4.6 ± 0.01 , 6.1 ± 0.05 , and 5.4 ± 0.05 , respectively. Table 2 lists the mean droplet size, PDI, and ZP values of both blank and medicated ME formulations. The droplet sizes of all the prepared ME formulations either blank or medicated are <20 nm with PDI values <0.38 . The small PDI values indicate that our prepared ME formulations are homogeneous and have a narrow droplet size distribution. This minuscule droplet size is very important to ensure good *in vivo* results. It greatly helps the transcorneal penetration of our ME formulations, as it is well-known that a droplet size less than 200 nm is required for accepted passive drug targeting through biological membranes.^{49,50} The obtained small droplet size of our ME was an expected outcome because of the presence of propylene glycol as a cosurfactant. This cosurfactant could penetrate the film formed by the surfactant mixture at the oil/water interface, lower its viscosity, and result in a microdroplets that have a smaller-radius curvature, which ultimately results in the production of transparent ME systems.^{43,51}

Concerning the zeta potential, sodium alginate and Carbopol 981 MEs, including both blank and medicated versions, possess large negative charges (>-26 mV). In contrast, chitosan MEs, including both blank and medicated versions, are positively charged. This difference in

the type of the charge is an inherent property of the polymers. Whatever its type, the surface charge of any colloidal system is very important factor to achieve a long period of shelf stability because it results in repulsion between droplets, which prevents their coalescence upon standing.

Transmission Electron Microscopy (TEM).

TEM analyses reveal the multilayered structure of the ME droplets, which have regular spherical outlines of miniscule droplet size (<20 nm) (Figure 2A). These results support the droplet size data obtained by the Zetasizer. Figure 2B illustrates a magnified ME droplet with clearly visible internal structures that show the presence of several nuclei of the internal aqueous phase suspended in the intermediate oil phase, which is surrounded by the extended external aqueous layer, exactly as predicted in the cartoon shown in Figure 2C.

Viscosity and Bioadhesion.

Figure 2D and E present the viscosity and bioadhesion force values of the prepared formulations containing 0.6% PRG. The tested formulations included ME formulations prepared using three different bioadhesive polymers, chitosan, sodium alginate, and Carbopol 981, compared to their control formulations that were prepared from the same polymers at the same concentrations in absence of the ME system. Formulations containing Carbopol 981, either in the presence or absence of ME system, have the highest viscosity compared to those prepared using sodium alginate or chitosan (Figure 2D). This difference in formulation viscosity may be attributed to the difference in the concentration, cross-linking, and molecular weight of the used polymer.⁵² Also, Figure 2D illustrates that two of three PRG ME formulations have significantly higher viscosity values compared to the corresponding control formulations that lack the ME ($p < 0.0001$ for both sodium alginate and Carbopol 981). The remaining PRG ME containing chitosan does not vary in viscosity from the polymer alone ($p = 0.1057$).

Regarding the bioadhesion of our formulations, chitosan-containing formulations possess the highest bioadhesion force compared to the formulations that contain the other polymers. There are several factors that may affect the bioadhesive force of the polymer including the ability of the polymer to swell, the extent of swelling, the polymer chain flexibility, and the type of binding with mucin.⁵³ The higher bioadhesion force of chitosan is likely attributed to its ability to interact with mucin via three different types of binding, H-bonding, hydrophobic association, and electrostatic binding due to its carried positive charge, which allow it to strongly bind to mucin. In contrast, sodium alginate and Carbopol 981 bind to mucin only via H-bonding.⁵⁴ Also, chitosan possesses high chain flexibility due to its moderate degree of acetylation (1525%), which allows it to adequately interact with mucin thus resulting in a higher bioadhesive force.^{55,56} Figure 2E illustrates that our PRG ME formulations have significantly higher bioadhesive forces compared to the corresponding control formulations that lack the ME ($p < 0.0001$, $p < 0.01$, and $p < 0.05$ for chitosan, Carbopol 981, and sodium alginate, respectively). The improved bioadhesion of our ME formulations may be attributed to the presence of nonionic surfactants, which improve polymer chains wetting and swelling and promotes their interaction with mucin, resulting in

better bioadhesion.⁵³ These results indicate that our ME PRG eye drops are expected to have a longer corneal contact time and longer duration of action after application to the eye.

X-ray Diffraction (XRD).

Figure 2F illustrates the XRD of PRG Carbopol 981 ME loaded on chitosan as a carrier, chitosan powder, and all the solid ingredients of the ME including Carbopol 981, lecithin, and PRG. The figure clearly demonstrates that all the solid ingredients of our ME, as well as the chitosan carrier, are amorphous powders. This is evidenced by the absence of sharp XRD peaks for all components with the exception of PRG, which shows distinct sharp peaks that reflect its crystalline structure. The disappearance of these specific PRG peaks in the XRD of our ME illustrates the absence of PRG crystals and confirms its presence in a completely soluble form.

In Vitro Release.

The *in vitro* release profiles of PRG-loaded ME formulations, as well as their control formulations, PRG aqueous solution, and PRG in different bioadhesive polymers: chitosan, sodium alginate, or Carbopol 981, are shown in Figure 2G. Comparison of the rate of PRG release from the ME formulations and their controls yields the following order: PRG in water > PRG in polymers > PRG ME. This *in vitro* release order is likely due to the free water solubility of PRG. When the drug is in aqueous solution, no additional steps are required for the drug to diffuse through the cellulose membrane, while an additional diffusion step through the viscous polymeric solution is required for the drug to first reach the membrane then diffuse through it. In contrast, movement through several transitions was required for PRG when in the ME form. This is described in greater detail in the following paragraphs.

Despite being a water-soluble drug that is characterized by a fast release behavior, our ME formulations succeed in sustaining the release of PRG up to 24 h, which is in contrast to control formulations that exhibit a rapid release of drug in the first few hours. Figure 2G illustrates that all ME formulations exhibit an initial fast release followed by a controlled release for the up to 24 h. Given the special engineering of our formulation, we fully anticipated this release behavior. Being multilayered with a limited volume of water in its innermost layer, our ME structure governs the drug distribution within the formulation. Because of the limited percentage of the internal Milli-Q water as an internal aqueous phase (20% of the W/O ME), only 40% of the drug dose is in the innermost layer of the ME, while the remaining 60% is incorporated in the polymeric outermost aqueous layer. The initial fast release is likely due to the presence of 60% of the drug dose in the external aqueous phase, which was ready to be released upon contact with the release medium after simple diffusion through the viscous polymeric external aqueous layer. In contrast, the presence of 40% of the drug dose in the innermost aqueous layer of the multiple W/O/W ME, which is surrounded by an intermediate layer of Labrafac oil, a nonsolvent for the drug, is responsible for the controlled release behavior that lasted up to 24 h.

Because of the multilayered engineering of our ME, to be released, PRG must pass through two interfaces, the inner W/O interface and the outer O/W interface, after which it must

diffuse through the viscous bioadhesive polymer of the external aqueous layer. Furthermore, because our drug is water-soluble and its solubility in Labrafac oil is negligible, the presence of the Labrafac oil layer may act to greatly prolong its release. Because of the combination of multiple layers with varying solubility profiles, the time required for the drug to pass through multiple layers is greatly prolonged. An additional reason for the prolonged release of PRG is the presence of drug in both the innermost and outermost layers of our W/O/W ME. The presence of the drug in the external layer may hinder or slow the drug release from the innermost layer by affecting the drug concentration gradient across the intermediate oil layer of our ME, which may result in an overall slow release behavior.⁵⁷

Comparison of the drug release rates from our ME formulations demonstrates that the PRG-loaded Carbopol 981 ME has the most sustained release behavior followed by sodium alginate ME, then chitosan ME. This difference in the release rates results from the difference in ME viscosities, as illustrated in Figure 2D. The higher the formulation viscosity, which is largely dependent on the polymer type and its concentration, the slower the release rate, which is caused by slow diffusion through the polymeric network structure.

Corneal Permeability.

To determine the transcorneal permeability of PRG from our ME formulations, as well as from the two controls, PRG aqueous solution and PRG in Carbopol 981 without ME system, we used fresh rabbit corneas excised from whole eyes of New Zealand white rabbits that were shipped overnight from Pel-Freez Biologicals in Hanks balanced salt solution over wet ice. To keep the cornea alive for the entirety of the 6-h experiment, we used BSS-PLUS irrigating solution in the receptor medium. This solution has a similar composition to aqueous humor and commonly used as a sterile intraocular irrigating solution due to its ability to maintain the anatomic and physiologic integrity of intraocular tissues.^{58,59}

As previously mentioned, PRG is a BCS class I drug that suffers from a rapid absorption leading to a short duration of action. The data shown in Table 3 demonstrate that our ME formulation can maintain the high permeability associated with PRG with no significant difference in the permeation rates (dM/dt), flux values (J), or permeability coefficients (P) compared to controls ($p = 0.4191$). These results also demonstrate that the addition of a bioadhesive polymer (Carbopol 981) into an aqueous solution of PRG has no significant effect on the drug permeability. Similarly, Chen *et al.*⁶⁰ reported that the addition of 0.75% Carbomer 940 has no significant effect on the permeation rate of triptolide from a ME.

Localization of CACNA2D1 in the Rabbit Eye.

Immunohistochemistry was performed to determine if the PRG-target protein, CACNA2D1, is expressed in eye tissues of Dutch belted rabbits that are associated with IOP modulation, similar to our previous finding in both mice and human donor eyes.⁵ Figure 3A, B, and C illustrate that CACNA2D1 is highly expressed in both the nonpigmented epithelium of the ciliary body and the trabecular meshwork where aqueous humor production and drainage occurs, respectively.

In Vivo Efficacy.

Our recently published data demonstrated that PRG (0.9% in 2% HPMC viscous eye drops) has the ability to decrease IOP in both C57Bl/6J mice ($28.6 \pm 3.5\%$) and Dutch belted rabbits ($22.1 \pm 2.8\%$). Unfortunately, the IOP returned to baseline after 6 h.⁵ To improve on these results, we sought to determine if our sustained release PRG ME could maintain IOP at lower level for longer period of time after a single drop application. Figure 3D illustrates the percent IOP reduction in Dutch belted rabbits after application of different PRG ME eye drops, PRG Carbopol 981 and PRG W/O ME without the external bioadhesive aqueous layer in addition to their blank formulations (*i.e.*, the same formulations without PRG). The calculated pharmacodynamic parameters are listed in Table 4. Regardless the type of polymer in the external phase, all prepared ME formulations induced a percent IOP reduction greater than 34% (Table 4). Among all the tested formulations, the PRG Carbopol 981 ME provided the maximum IOP-lowering effect ($42.3 \pm 2.6\%$ reduction in IOP) that returned to baseline at 32.7 ± 1.3 h after application ($AUC = 788.6 \pm 36.8\text{h}$). This is a marked improvement compared to the control formulation (PRG in Carbopol 981 in absence of ME), which produced only a $29.4 \pm 1.4\%$ IOP reduction that returned to baseline at 9.3 ± 0.7 h ($AUC = 172.3 \pm 16.9\text{ h}$). The significant improvement in efficacy and duration of effect strongly suggests that our Carbopol 981 ME could be used as a once daily IOP-lowering therapeutic.

Statistical analysis of different pharmacodynamic parameters (Table 5) illustrates that there is no significant difference in either percent IOP reduction or $T_{\max}$ values between all the tested formulations including controls ($p = 0.0818$ and 0.473 , respectively). This nonsignificant difference between all the tested formulations may be due to the presence of free drug that was available to produce a rapid onset that reach its maximum after 3 h. In contrast, the T_{end} values of our ME eye drops were significantly longer compared to the control formulation ($p < 0.0001$). This is likely due to the ability of our ME formulation to sustain PRG release and prolong its corneal contact time that make the ME formulation an ideal for once daily administration.

The IOP-lowering results of our ME formulations demonstrate that we have succeeded in achieving our goal of producing bioadhesive formulations with rapid IOP-lowering onset and long duration of action. The main cause of the rapid onset was the presence of 60% of the drug dose in the external aqueous phase of the ME, while the presence of the remaining 40% in the innermost aqueous layer is responsible for the long duration of action that lasted for 32.7 ± 1.3 h for the Carbopol 981 ME. Upon comparison of the IOP-lowering effects of the three tested MEs, the order of their efficacy is as follows: Carbopol 981 ME > sodium alginate ME > chitosan ME. The main cause of this order may be the difference in their viscosities (Figure 2D). The high viscosity of Carbopol 981 ME and its' *in situ* gelling ability at physiological pH⁶¹ likely prevented its drainage from the eye and allowed enough time for the bioadhesive interaction to occur. In addition, the Carbopol 981 ME has the slowest release rate, which together with its high viscosity and bioadhesion allows for the longer duration of action. Although chitosan ME has the highest bioadhesive force (Figure 2E), and it has the lowest IOP-lowering capacity among our MEs (T_{end} and AUC ; Table 4). This might be due to its low viscosity that may allow some of the formulation to drain from

the surface of the eye before being strongly adhered to the ocular surface. Also, the lower pH value of our chitosan ME may be the cause of its shorter duration of action. Immediately upon chitosan ME installation into rabbit eyes, we observed a slight transient tearing, which could lead to a partial washing out of the formulation from the eye surface, thus possibly affecting the duration of its IOP-lowering effect.

When comparing Carbopol 981 ME and the control formulation containing Carbopol 981 only as a polymer at the same concentration, the ME possessed better IOP-lowering capacity and longer duration of action (Figure 3D and Table 4). This may be due to the higher bioadhesive force of the ME formulation due to its content of nonionic surfactants that have the ability to improve the polymer chain wetting and swelling, which could promote its interaction with the mucin layer that covers the eye surface resulting in stronger bioadhesion and longer duration of action.⁵³

Comparison of the IOP-lowering effect of W/O ME with that of W/O/W MEs effectively demonstrates that the presence of the bioadhesive external aqueous layer is very important for the prolonged IOP-lowering effect of our formulation. Table 4 illustrates that in the presence of the external aqueous layer, the duration of the IOP-lowering effect of our MEs ranged between 20 and 32.7 h, and the AUC ranged between 323 and 788.6% h. In contrast, in the absence of the external aqueous layer, the duration of the IOP-lowering effect was only 7.33 h with AUC of 98.9% h. The decreased parameters may be due to the rapid drainage of the formulation due to the lack of bioadhesive reaction.

Safety and Biocompatibility after Single Dose.

Regarding the short-term safety of our ME eye drops, slit-lamp biomicroscopic examinations performed when IOP returned to baseline levels after a single drop of ME, demonstrate that there is no sign of irritation such as redness or swelling. The cornea is clear with a smooth epithelial surface. There is no flare in aqueous humor, and the lens is transparent (Figure 4A).

The results of the *in vitro* cytotoxicity assay of our PRG-loaded Carbopol 981 ME and controls at different time points (1, 2, 3, and 6 h) are shown in Figure 4B. The results illustrate that the cell viability of our tested formulation is significantly different from that of the positive control (1% Triton-X 100) at all the time points ($p < 0.0001$), while it is equivalent to the negative control (untreated cells) ($p > 0.05$). Similarly, when tested separately, neither PRG nor the blank ME is cytotoxic to human corneal limbal epithelial cell line (HCLE) cells⁶² (data not shown). Typically, 15 min of incubation is sufficient to evaluate cell toxicity of conventional topical ocular formulations, which are eliminated from the eye surface within 5 min.⁶³ However, longer incubation times up to 4 h are used when the formulation contains bioadhesive polymers,⁶³ which is used to simulate long-term therapy. Taking into consideration the strong bioadhesion and the long contact time of our ME formulation, we extended the time frame of our cell viability studies to 6 h and demonstrated no reduction in cell viability, which further supports the biocompatibility of our ME.

Safety, IOP-Lowering, and Drug-Tissue Biodistribution Studies after Prolonged Use.

Because glaucoma is a chronic disease, our ME formulation has to be safe during prolonged use. Figure 5A and B present slit-lamp biomicroscopic examination images of Dutch belted rabbit eyes after 21 consecutive days of topical application of 100 μL of medicated (PRG Carbopol 981 ME) and blank (Carbopol 981 ME) formulations, respectively. It is obvious that corneas are clear and smooth, aqueous humor is free of cells or flare, and lenses are clear and free from any abnormality. Collectively, these data support the safety of our ME after prolonged use. The safety of our formulation is also confirmed by the corneal histological studies obtained after the slit-lamp biomicroscopic examinations (Figure 5C–F). The histological images show that the corneal structure is intact and its architecture is maintained. Also, there is no any sign of abnormality. All corneal layers have full thickness and appear healthy. This excellent safety upon prolonged use may be due to the safety and biocompatibility of the used ingredients.^{39,42,43,45,46}

Figure 5G presents the IOP profile of the rabbits over 21 days of the study and demonstrates that our formulation is able to maintain the IOP at low value using only a single daily dose without the development of any observed side effects, irritation, resistance, or decrease in the drug response upon prolonged use. Figure 5H and I show the drug ocular tissue biodistribution after 21 days of single daily dosing for the medicated and the blank Carbopol 981 ME, respectively. It is evident that the treated eye accumulated high concentrations of the drug distributed in various compartments of the eye, while the other eye that received the blank ME only accumulated a miniscule amount of pregabalin. This behavior may be due to the bioadhesion of the ME, which localized the drug within the treated eye and limit its systemic absorption. In support of this, we detected miniscule drug concentrations within peripheral organs such as lungs and brain and the complete absence of drug within other peripheral organs such as liver, kidneys, spleen, heart, and plasma (data not shown).

Physical and Chemical Stability.

Because the storage conditions of our PRG-loaded Carbopol 981 ME eye drops are completely unknown, we evaluated its physical and chemical stability for one month at different temperatures (5 °C, 25 °C, 30 °C, and 40 °C). After one month of storage, no signs of physical instability (creaming, turbidity, phase separation, or precipitation) were observed, thus demonstrating the high physical stability of our ME eye drops. Similarly, there were no significant changes in pH, droplet size, polydispersity index, zeta potential, or drug content of our PRG-loaded Carbopol 981 MEs compared to freshly prepared formulation (Figure 6). Collectively, these results confirm the high stability of our ME eye drops for one month at temperatures ranging from 5 to 40 °C.

CONCLUSION

Glaucoma is the leading cause of irreversible blindness worldwide. Elevated IOP is the most significant risk factor for visual field loss in POAG, the most common form of glaucoma. Preservation of IOP within physiologically normal limits is a very important strategy toward management of POAG and prevention or slowing down of disease progression. Because of the importance of a tightly maintained IOP, coupled with the fact that IOP can be medically

controlled, IOP reduction is the first-line therapeutic option in glaucoma. However, many current IOP-lowering drops suffer from poor patient compliance due to their short half-life and low residence time on the cornea, which require topical application multiple times per day. Although several glaucoma therapeutics are described as once daily, they still suffer from multiple and sometimes significant side effects. These gaps represent enormous unmet needs.

In this study, we succeeded in addressing many of the limitations associated with current drug therapies by developing a biocompatible nontoxic lead formulation that is optimized for once daily topical ophthalmic use. To accomplish our goal, we prepared multiple water-in-oil-in-water (W/O/W) MEs that took advantage of both ME types: the slow release behavior associated with the water-in-oil (W/O) type; and the aqueous sensation of the oil-in-water (O/W) type. Our lead ME formulation is capable of prolonging corneal contact time because of the presence of a bioadhesive polymer in the external aqueous phase. It provides for controlled drug release due to the presence of an intermediate oil layer, which is a nonsolvent for the drug. The multilayered structure of our formulation also maintains the high corneal permeability of the native drug. Because of this engineering, our formulations demonstrate a sustained drug release that lasts for up to 24 h. Because of the distribution of drug between the inner and outer aqueous phases, after application to the eye, our Dutch belted rabbit test subjects experienced a rapid onset followed by a prolonged phase of sustained IOP reduction. The IOP-lowering response was maintained during 21 days of daily dosing and our formulation exhibited high biocompatibility. We conclude that our PRG ME eye drops could serve as a promising once daily glaucoma therapeutic and drug delivery system with excellent physical and chemical stability.

METHODS

Materials.

PRG (97% purity), poloxamer 188 (pluronic F-68), sodium phosphate dibasic, sodium bicarbonate, chitosan (low molecular weight, 75–85% deacetylated), potassium chloride, potassium dihydrogen phosphate, calcium chloride dihydrate, dipotassium hydrogen phosphate, glacial acetic acid, Triton X-100, Cremophore EL, methyl thiazol tetrazolium (MTT), and glutathione disulfide were purchased from Sigma-Aldrich (St. Louis, MO). Propylene glycol, magnesium chloride hexahydrate, dextrose, Tween 80, sodium chloride, gastric mucin (type II), dimethyl sulfoxide (DMSO), phosphoric acid, methanol, and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ). Gift samples of Labrafac Lipophile WL1349, Capryol 90, transcutol P, and Labrasol were obtained from Gattefossé Corporation (Paramus, NJ). Soybean L- α -Lecithin (97.7% phosphatidyl choline) was purchased from Calbiochem (Billerica, MA). Ethyl alcohol was purchased from Decon Laboratories, Inc. (King of Prussia, PA). Sodium alginate (viscosity of 1% solution at 25 °C = 5–40 cP) was purchased from MP Biomedicals (Solon, OH). Carbopol 981 was obtained as a gift sample from Lubrizol Advanced Materials, Inc. (Cleveland, OH). Keratinocyte-SFM serum free medium was purchased from Life Technologies Corporation (Grand Island, NY). DMEM/F-12 (Dulbecco's modified Eagle's medium/nutrient mixture F-12) was purchased from Mediatech, Inc. (Manassas, VA). Fresh

whole eyes of male New Zealand white rabbits were obtained from Pel-Freez Biologicals (Rogers, AR).

Animals.

Dutch belted male rabbits (1.5–2.5 kg) procured from Covance Inc., (NJ, USA) were used to test IOP-lowering effects of our formulations. All procedures including rabbits were approved by the Animal Care and Use review board of the University of Tennessee Health Science Center (UTHSC) and followed the Association of Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research in addition to the guidelines for laboratory animal experiments (Institute of Laboratory Animal Resources, Public Health Service Policy on Humane Care and Use of Laboratory Animals).

HPLC Analysis of PRG.

A previously published reversed phase HPLC method was used for quantification of PRG through all experiments.⁶⁴ An Agilent 1100 series HPLC system (Germany) was attached to Supelco kromasil C18 column (5 μ m, 100 Å, 4.0 mm $\times$ 300 mm). The mobile phase consisted of a mixture of methanol/acetonitrile/0.02 M dipotassium hydrogen orthophosphate (3:1:16, v/v/v) at a flow rate of 1 mL/min. The effluent was monitored by photodiode array detector, and PRG was detected after a retention time of 5.4 min at a detection wavelength of 210 nm.

Solubility Determination of PRG.

Solubility screening of PRG in different media as well as the ingredients of our ME was carried out to determine its maximum solubility in each ingredient. The used media included: Labrafac oil, (Capryol 90 and soybean lecithin, 1:1 weight ratio mixture), Milli-Q water, 0.01N HCL, and 0.01N NaOH. An excess amount of the drug was added to 2 mL of each medium in screw capped glass bottles. Suspensions were shaken at 180 rpm for 7 days in a thermostatically controlled shaker at 25 ± 0.5 °C. The obtained suspensions were allowed to equilibrate further for 1 day. After equilibration, suspensions were filtered through 0.45 μ m membrane filters (Milli-Q, Billerica, MA) and the filtrate was suitably diluted with the mobile phase then assayed for its drug content by HPLC. In case of oil and surfactant media, the filtrate was diluted with absolute ethanol before HPLC assay. Each experiment was performed in triplicate, and the results were calculated as mean $\pm$ SEM.

Preparation of Multiple ME Eye Drops.

Preparation of our multiple W/O/W ME eye drops was achieved in three steps. The first step included the formation of the primary W/O ME. The second step involved the preparation of the bioadhesive external aqueous phase. Finally, the primary W/O ME was further emulsified into the bioadhesive external aqueous phase to produce the final bioadhesive multiple W/O/W ME eye drops.

Construction of Ternary/Pseudo-Ternary Phase Diagrams and Preparation of Primary W/O ME.—The primary W/O ME usually consists of an oil phase, aqueous

phase, and single surfactant or surfactants mixture (HLB less than 7). To determine the appropriate ratio of each component that can efficiently produce a ME, we constructed several ternary phase and pseudo-ternary phase diagrams using Labrafac oil as the oil phase, Milli-Q water as an aqueous phase, and a single surfactant (in case of ternary phase diagrams) or surfactant mixture (in case of pseudo-ternary phase diagrams). The used surfactants were Capryol 90, transcutool P, soybean lecithin, or different combination of them.

The ternary phase diagrams were generated using a water titration method.^{65,66} In this method, Labrafac oil was mixed with surfactant or surfactants mixture in different ratios by vortex for 2 min. The produced mixture was then titrated with Milli-Q water until the appearance of the first turbidity, which reflected the boundary point that differentiated the end of the ME region and the beginning of macroemulsion region. Ternary or pseudo-ternary phase diagram that showed the largest ME area was selected to pick up one point to prepare the primary W/O ME to be used for further investigations. The picked-up point consisted of 20% Milli-Q water +30% Labrafac oil +50% (Capryol 90 and soybean lecithin, 1:1 mixture). Forty percent of the PRG dose was incorporated in the aqueous phase of the primary W/O ME due to its limited volume.

Preparation of External Aqueous Phase and Incorporation of Bioadhesive Polymers.—During the preparation of the external aqueous phase, several surfactants with high HLB values were screened such as Tween 80, Cremophor EL, poloxamer 188, brij 97, and Labrasol. Aqueous solutions of different concentrations of these surfactants were prepared and tested by titration with the previously prepared primary W/O ME until the appearance of the first turbidity. The surfactant solution that could incorporate the greatest percentage of the primary W/O ME was selected for further investigations. To increase the percentage of incorporated W/O ME, the selected surfactant was mixed with other surfactants or cosolvent.

After surfactant screening, the external aqueous phase was prepared by dissolving 7.4% Labrasol, 7.4% Cremophor EL, and 22.2% propylene glycol in Milli-Q water (Table 1). The remaining amount of the PRG dose (60%) was dissolved in the external aqueous phase of the final W/O/W. The bioadhesive polymers, chitosan (1.1%), sodium alginate (0.4%), or Carbopol 981 (0.15%), were soaked and allowed to swell overnight in the previously prepared external aqueous phase in which 60% of the PRG dose was incorporated (1% acetic acid was used instead of Milli-Q water in case of chitosan ME).

Preparation of Final W/O/W ME.—For preparation of the final formulation, the previously prepared drug-loaded primary W/O ME was incorporated dropwise into the drug-loaded external aqueous polymeric solution and gently mixed until a clear final product was obtained.

***In Vitro* Evaluations.**

Measurement of pH.—The pH values of each ME formulation were measured using a pH meter (Corning pH meter 440; Corning Inc., Corning, New York). One gram of each

formulation was dispersed in 20 mL of Milli-Q water, and then the pH was measured. The experiment was repeated three times, and the results were presented as mean $\pm$ SEM

Average Droplet Size, Polydispersity Index (PDI), and Zeta Potential

Measurement.—The average droplet size, PDI, and zeta potential of our ME formulations were determined after suitable dilution using Zetasizer (Nanoseries, nano-ZS, Malvern Instruments Limited, UK).⁶⁷ All measurements were performed at 25 °C. The results of three independent test runs were presented as mean $\pm$ SEM.

Transmission Electron Microscopy.—The morphology of our PRG Carbopol 981 ME eye drops was examined using transmission electron microscopy (TEM) (JEOL JEM1200EX II electron microscope). Briefly, the ME formulation was diluted 1:100 with Milli-Q water. Two microliters of the diluted ME was placed on 400 mesh copper grids covered with Formvar film (Electron Microscopy Sciences EMS, Hatfield, PA). The grids were allowed to dry for 2 h in a desiccator followed by negative staining with Uranyless EM stain (Electron Microscopy Sciences EMS, Hatfield, PA) before examination by TEM.

Determination of Viscosity.—A cone (1.5°) and plate rotary viscometer (Brookfield DV-II+ programmable viscometer; Brookfield Engineering Laboratories, Middleboro, MA) was used to determine the viscosity of our formulations according to our previously published protocol.^{68,69} Each formulation (500 μ L) was placed on the stationary plate of the viscometer for 5 min before each measurement to reach the running temperature. The viscosity was measured in triplicate at 35 $\pm$ 0.5 °C, and the results were calculated as mean $\pm$ SEM.

Measurement of Bioadhesive Force.—The bioadhesive force of our ME eye drops was determined by a simple method that depends on evaluation of the rheological synergism that happen upon mixing the bioadhesive polymer with mucin dispersions.^{70,71} Gastric mucin type II (15%, w/v) was dispersed in simulated tear fluid (pH 7.4) and allowed to dissolve overnight at 4 °C. Immediately before measurement, mucin dispersion was warmed to 35 °C and then mixed with the formulations that had been warmed to the same degree. The viscosities of mucin dispersion, formulations, and their mixture were measured in triplicate using the Brookfield viscometer. Viscosity change due to bioadhesion as well as the bioadhesive forces was calculated by employing the following equations, and the results were represented as mean $\pm$ SEM:^{70,71}

$$\eta_b = \eta_t - (\eta_m + \eta_p) \quad (1)$$

$$F_b = \eta_b \times Y \quad (2)$$

where η_b is the viscosity due to bioadhesion, η_t is the viscosity of the mixture, η_m is the viscosity of mucin, η_p is the viscosity of the formulations, F_b is the bioadhesive force, and γ is the shear rate at which the viscosity value was measured.

X-ray Diffraction (XRD).—Freedom from any crystalline particulate material is a very important criterion that should be taken into consideration to ensure the safety and stability of our formulation. For this reason, XRD examination was performed on our PRG Carbopol 981 ME loaded on chitosan powder as a carrier, chitosan powder alone, and all the solid ingredients of our PRG Carbopol 981 ME. XRD patterns of the samples were recorded at room temperature on AXS D8 Advance X-ray Diffractometer (Bruker, Germany) using Cu-K α radiation and at a power of 40 kV and 40 mA. The data were recorded over a scanning 2θ range of 2° to 60° .^{72,73}

In Vitro Drug Release.—PRG release behavior from our formulations was studied according to our previously published protocol.^{74,75} Briefly, we used 1500 μ L fast microequilibrium dialyzers to which a semipermeable regenerated cellulose membrane with molecular weight cutoff of 5000 Da attached (Harvard Apparatus Co., Holliston, MA). One-hundred microliters of our formulations or the controls was placed in the donor chamber. The used control formulations include 0.6% PRG aqueous solution or 0.6% PRG viscous solution using different polymers (chitosan, sodium alginate, and Carbopol 981). Warmed PBS pH 7.4 (1.5 mL at $35 \pm 0.5^\circ\text{C}$) was placed in the receptor chamber. The dialyzer was kept in thermostatically controlled shaker ($35 \pm 0.5^\circ\text{C}$ and 50 rpm). At predetermined time intervals ranging from 0.25 to 24 h, the entire medium in the receptor chamber was withdrawn and replaced by 1.5 mL of fresh warmed medium. The concentration of released drug was then determined by HPLC.

The drug content of each formulation (100%) was measured, allowing for an accurate calculation of the cumulative percent of PRG released based on its actual drug content. One-hundred milligrams of each formulation was accurately weighed in a stoppered volumetric 10 mL measuring flask. Milli-Q water was added to bring to volume of 10 mL in control formulations. For ME eye drops, a combination of Milli-Q water and absolute ethyl alcohol (3:7) was added. Absolute ethyl alcohol was used to dissolve water-insoluble ingredients of the ME. The flask was shaken for 10 min, and the solution was filtered using 0.22 μ m membrane filters (Millipore, Billerica MA) and then assayed for the total PRG content by HPLC using the method described before. The cumulative percent amount released of PRG was calculated as mean $\pm$ SEM based on the actual measured drug content. All experiments were performed in triplicate.

The data were statistically analyzed using one-way analysis of variance (ANOVA) test followed by Tukey's multiple comparisons test. Statistical calculations were carried out using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA).

Corneal Permeability.—Modified rounded junction Franz diffusion cells (PermeGear Inc.) were used to study the PRG corneal permeability. Corneas were mounted to the cells with the epithelial side facing the donor chamber containing the formulations.⁷⁶ The temperature of the cells was maintained at $35 \pm 0.5^\circ\text{C}$ with the help of a circulating water bath. One-hundred microliters of PRG formulations, chitosan ME, sodium alginate ME, and Carbopol 981 ME, in addition to the two controls, PRG aqueous solution and PRG in Carbopol 981, was placed in the donor chamber. The receptor chamber was continuously stirred and filled with 5 mL of balanced salt solution enriched with bicarbonate, dextrose,

and glutathione, BSS-PLUS (Alcon Laboratories Inc., Fort Worth, TX). At predetermined time intervals (1, 2, 3, 4, 5, and 6 h), 500 μL were withdrawn from the receptor chamber and replaced with an equal volume of fresh warmed BSS-PLUS. The drug concentration in withdrawn samples was immediately determined by HPLC assay as described above. The results were plotted as cumulative amount permeated (μg) *versus* time. Also, the steady-state flux (SSF) was calculated by dividing the rate of transport at steady state by the surface area of the cornea through which permeation occurred. Flux (J) as well as permeability coefficient (P) was calculated using the following equations:⁷⁶

$$\text{Flux } (J) = (dM/dt)/A \quad (3)$$

$$\text{Permeability } (P) = \text{Flux} / C_d \quad (4)$$

where M is the cumulative amount of drug transported, A is the surface area of the corneal membrane (0.636 cm^2) exposed to the drug, and C_d is the initial drug concentration in the donor chamber.

Immunohistochemistry.

To immunolocalize the PRG target protein, CACNA2D1, in Dutch belted rabbits' paraffin-embedded eye sections ($50 \mu\text{m}$ thickness), we used our previously published methods.^{5,77} Briefly, we used 10% goat serum to block nonspecific binding sites in tissue sections. An anti-CACNA2D1 monoclonal antibody (ThermoFisher Scientific, Rockford, USA, catalog # MA3-921; 1:100) was used in our study as the primary antibody along with donkey antimouse Alexa fluor 488 secondary antibody (Invitrogen, Waltham, MA; catalog #A21202; 1:200) and DAPI (Vector Laboratories, Inc., CA; catalog #H-1200) as a nuclear counterstain. Vector TrueVIEW Autofluorescence Quenching Kit (Vector Laboratories, Inc., CA; catalog #SP-8400) was used to remove the unwanted background autofluorescence in our sections. Sections were viewed and images were obtained using a Nikon C1 confocal microscope (Nikon, NY).

In Vivo Evaluations.

Formulations Safety.—The safety of ME eye drops was tested by installation of 100 μL of the medicated formulation in the lower conjunctival sac of the right eye of Dutch belted rabbits ($n = 3$), while the left eye served as a control. Eyes were examined every hour for any sign of irritation such as redness, tearing, and conjunctival or corneal swelling.⁷⁸ Slit-lamp examinations were performed on the eyes of all rabbits after the IOP returned to baseline.⁷⁸

Formulations Efficacy.—The IOP-lowering effect of our formulations was determined using Dutch belted rabbits ($n = 3$). During the study, 100 μL of the ME eye drops was applied into the inferior conjunctival sac of the right eye of the Dutch belted rabbits, while the left eye served as a control by receiving 100 μL of the blank formulation. The IOP was measured using a Tono-pen AVIA (Reichert Technologies, Depew, NY) immediately before application of the formulation (baseline) and at predetermined time intervals until it returned back to its baseline value.⁵ Five consecutive IOP readings were averaged for each individual eye at each measurement.

Evaluation of PRG formulations was based on comparing the calculated pharmacodynamic parameters including maximum percent reduction in IOP [IOP reduction (%)], 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}), and the total area under the % IOP reduction-*versus*-time curve (AUC). All results were expressed as mean $\pm$ SEM.

***In Vitro* Evaluation of Formulation Cell Toxicity.**

In vitro cytotoxicity of our PRG-loaded Carbopol 981 ME eye drops (this formulation was selected based on *in vivo* results) was evaluated by the MTT assay using a modified previously published protocol.⁷⁹ The assay was carried out in 96-well plates (Costar 3596, Corning Inc., Corning, NY). HCLE was seeded at a concentration of 18 000 cell/well and incubated in a humidified environment at 37 °C in 5% CO₂ for 24 h in Gibco-Keratinocyte-SFM medium (1X) (plus bovine pituitary extract, epidermal growth factor and CaCl₂·2H₂O). After 24 h, the medium was replaced by 50 μL of artificial tears, and then 150 μL of the diluted formulations (2.15 μL formulation +147.85 μL medium) was added slowly above the artificial tears. This dilution was based on surface area of the cell layer in the well exposed to the formulation compared to the actual rabbit corneal surface area. In addition, after different time points of incubation (1, 2, 3, and 6 h), the formulations were removed and the plate was washed with the culture medium to remove all the traces of the formulations. Two-hundred microliters of MTT reagent (1 mg/mL in culture medium) was added to each well, and the plates were incubated at 37 °C for 4 h. After incubation, the MTT was removed, and 200 μL of DMSO was added to each well to dissolve the formazan crystals. The plate was then shaken for 15 min. The absorbance of the solution was measured at 570 nm and converted to percent cell viability relative to the negative control (untreated cells) by a μ -Quant universal microplate spectrophotometer (Bio-Tek Instruments, Inc. Winooski, VT). Statistical analysis of the percent cell viability data was performed using a one-way ANOVA followed by Tukey's multiple comparisons test. Each experiment was performed in eight replicates and the results expressed as mean $\pm$ SEM.

Safety, IOP-Lowering, and Drug-Tissue Biodistribution Studies after Prolonged Use.

To evaluate the safety and efficacy of our ME formulation for an extended period, we monitored IOP for 21 days to ensure its suitability for prolonged use. Male Dutch belted rabbits ($n = 5$) were used for such study. Each rabbit received a daily dose of 100 μL of PRG Carbopol 981 ME in the lower conjunctival sac of one eye while receiving the blank formulation in the other eye for 21 successive days. IOP of both eyes was measured as previously mentioned under the formulation efficacy study. The IOP was measured twice daily, one time immediately before the formulation application and the second measurement done at $T_{\max}$. After 21 days, all rabbits were subjected to a slit-lamp biomicroscopic examination to evaluate any signs of irritation or ocular damage. Rabbits were euthanized, and both eyes, as well as some internal organs (heart, lungs, kidneys, liver, spleen, brain, and plasma), were collected. Eyes were dissected into different tissues, while fresh and all the tissues were kept at -80 °C until assayed for their drug contents. Each cornea was divided into two halves, one of which was used to estimate its drug content, while the other was subjected to histological examination to detect any abnormality in the corneal tissues as a result of the daily ME application. The drug contents of the tissues were evaluated using

LC–MS/MS method with a limit of quantitation of 2.5 pg/mL.⁸⁰ Briefly, tissues were homogenized in a frozen methanol using tissue tearor (Biospec. Inc., Bartlesville, OK). Tissue homogenates were kept overnight at –20 °C to dissolve pregabalin and precipitate protein. The methanolic extract was separated by centrifugation at 14 000 rpm for 15 min at –5 °C and then evaporated until dryness, and the residue was dissolved in the mobile phase before being injected in the LC–MS/MS system (SCIEX-5500).⁸⁰

Physical and Chemical Stability.

To determine the physical stability of our PRG-loaded Carbopol 981 ME eye drops, we prepared three batches of formulation. ME eye drops were placed in white dropper bottles under aseptic conditions, which were then stored in thermostatically controlled air ovens at four different temperatures (5 ± 3 °C, 25 ± 2 °C, 30 ± 2 °C, and 40 ± 2 °C⁸¹) and ambient humidity for one month. In addition, to easily monitor any change in the physical appearance of our ME eye drops, our formulation was also placed in a transparent glass bottles (7 mL) at each studied temperature. The tested formulations were evaluated initially and after one month for pH, droplet size, PDI, zeta potential, and drug content. Each experiment was performed in triplicate, and the results were calculated as mean $\pm$ SEM. Data were statistically analyzed using one-way analysis of variance (ANOVA) test compared to the initial parameters before the stability study was conducted.

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Figure 1.

Ternary and pseudo-ternary phase diagrams of the primary W/O ME. The primary W/O ME was formed of Labrafac oil/Milli-Q water and surfactant or surfactant mixture as follows: (A) Capryol 90; (B) Capryol 90/lecithin [1:1]; (C) Capryol 90/lecithin [1:2]; (D) Capryol 90/lecithin [2:1]; (E) Transcutol P; and (F) Transcutol P/lecithin (1:1). The shaded regions represent the W/O ME area. Panel B was chosen to select one point to prepare our W/O ME because it had the largest shaded area, thus giving us the largest number of possible combinations for selection.

Figure 2.

(A) TEM image of diluted PRG-loaded ME negatively stained by Uranyless EM stain revealed the multilayered droplet structure with spherical outlines of miniscule droplet size (<20 nm) that are scattered within an expanded external aqueous layer that has been diluted with water. (B) Magnified ME droplets consist of several nuclei of the internal aqueous phase entrapped in the intermediate oil phase, which is surrounded by the external aqueous layer. (C) Cartoon illustrating the multilayered structure of the ME. (D) Viscosity (cP) and (E) bioadhesive force in dyn/cm^2 of PRG-loaded ME formulations and controls were calculated as mean $\pm$ SEM; $n = 3$. Formulations containing Carbopol 981 either in the presence or absence of ME system have the highest viscosity values compared to those contained chitosan or sodium alginate. However, chitosan-including formulations possess the highest bioadhesive force compared to the formulations that include the other polymers. (F) X-ray diffraction graphs of PRG Carbopol 981 ME loaded on chitosan as a carrier,

chitosan powder, and the solid ingredients of the ME. The figure demonstrates that our ME did not show any specific peaks, which confirms the absence of any crystal form and also confirms that PRG and other solid contents of ME are completely soluble. (G) Cumulative amount of PRG released (%) from ME formulations and controls (mean $\pm$ SEM; $n = 3$). PRG Carbopol 981 ME formulation succeeded in slowing and sustaining the release of PRG up to 24 h in contrast to the control formulations that exhibited rapid release of the entire drug in the first few hours. PRG, pregabalin; ME, microemulsion.

Figure 3.

(A) Localization of PRG target protein, CACNA2D1, in the eye of a Dutch belted rabbit. CACNA2D1 (green) is localized to the trabecular meshwork [higher magnification is shown in (B)] and the ciliary body [higher magnification is shown in (C)]. Blue = nuclei, TM = trabecular meshwork, CB = ciliary body. (D) Percentage IOP reduction/time profiles after topical application of a single dose of different PRG-loaded formulations to the right eyes and the blank formulations to the left eyes of Dutch belted rabbits (mean $\pm$ SEM; $n = 3$). PRG Carbopol 981 ME provided the maximum IOP-lowering effect ($42.3 \pm 2.6\%$ reduction in IOP) that returned to baseline at 32.7 ± 1.3 h after application. In comparison, PRG in Carbopol in absence of ME system produced a $29.4 \pm 1.4\%$ IOP reduction that returned to baseline at 9.3 ± 0.7 h. These data demonstrate that the presence of the bioadhesive external aqueous layer is very important to produce a deep and prolonged IOP-lowering effect; PRG

W/O ME that lacks the external aqueous phase produces an IOP reduction of only 23.8 ± 1.9 IOP that returned to baseline at 7.33 ± 1.33 h.

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Figure 4.

(A) Slit-lamp examination of Dutch belted rabbit eyes showed that the cornea and lens were clear with no signs of irritation after application of a single dose of our ME eye drops, chitosan ME, sodium alginate ME, and Carbopol 981 ME. (B) Histogram of cytotoxicity of PRG-loaded Carbopol 981 ME formulation using HCLE (mean $\pm$ SEM; $n = 8$) demonstrated no cytotoxicity to HCLE cell line at the different studied incubation periods (1, 2, 3, and 6 h). PRG = pregabalin, ME = microemulsion.

Figure 5.

(A, B) Slit-lamp biomicroscopic examination of rabbit eyes after 21 days of daily dosing with Carbopol 981 ME medicated and blank formulations, respectively. Both photos show that the cornea is smooth, the aqueous humor is clear, and the lens appears normal, which collectively demonstrate the safety of our formulation during prolonged use. Images of histological examination of rabbit corneas after 21 days of daily dosing of (C, D) Carbopol 981 ME medicated and (E, F) blank formulations showed normal corneal architecture. (G) IOP profiles of Dutch belted rabbits for 21 days of daily dosing of Carbopol 981 ME medicated and blank formulations show that our formulation does not suffer from any resistance or decrease in the drug response during prolonged use. (H, I) Drug tissue biodistribution after 21 days of daily dosing of Carbopol 981 ME medicated and blank formulations, respectively, show that PRG is distributed though different tissues of the treated eye, while the control eye dosed with the blank ME only accumulates a miniscule

amount of PRG. Epi, corneal epithelium; Endo, corneal endothelium; Eye cup, consists of sclera, choroid, retinal pigmented epithelium, ciliary body, and trabecular meshwork.

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Figure 6.

(A) pH, (B) average droplet size, (C) polydispersity index, (D) zeta potential, and (E) drug content of PRG-loaded Carbopol 981 ME eye drops after storage for one month at different temperatures (mean $\pm$ SEM; $n = 3$). There was no significant difference in any of the studied parameters ($p > 0.05$) compared to the initial values obtained from freshly prepared formulation.

Table 1.Composition of PRG-Loaded Bioadhesive Multiple W/O/W ME^a

ME ingredients (%W/W)	
PRG	0.6
Labrafac oil	7.8
Capryol 90	6.5
Lecithin	6.5
Labrasol	7.4
Cremophor EL	7.4
Propylene glycol	22.2
Bioadhesive polymer	X ^b
Milli-Q water to	100

^aW/O/W ME: water-in-oil-in-water microemulsion.^bX: Chitosan = 1.1%W/W, sodium alginate = 0.4%W/W, or Carbopol 981 = 0.15% W/W.

Table 2.

Droplet Size, PDI,^a and Zeta Potential of Blank and Medicated ME^b Formulations^c

type of ME eye drops	mean droplet size (nm)		PDI		zeta potential (mV)	
	blank	medicated	blank	medicated	blank	medicated
PRG ^d chitosan ME	17.4 ± 0.0	17.4 ± 0.2	0.36 ± 0.0	0.37 ± 0.0	15.9 ± 1.3	10.2 ± 0.8
PRG sodium alginate ME	16.8 ± 0.2	16.5 ± 0.1	0.34 ± 0.0	0.34 ± 0.0	-26.8 ± 0.6	-26.7 ± 1.0
PRG Carbopol 981 ME	16.0 ± 0.1	15.4 ± 0.0	0.26 ± 0.0	0.26 ± 0.0	-30.1 ± 1.3	-26.3 ± 0.6

^aPDI: polydispersity index.

^bME: microemulsion.

^cData are expressed as mean ± SEM; *n* = 3.

^dPRG: pregabalin.

Table 3.

In Vitro Transcorneal Permeability Parameters of PRG from Multiple W/O/W MEs^a and Controls^b

formulation	rate of permeation (dM/dt)	flux ($\mu\text{g}/\text{cm}^2/\text{min}$)	permeability coefficient (P) $\times 10^4$ (cm/min)
PRG ^c in water	0.285 $\pm$ 0.04	0.45 $\pm$ 0.06	7.5 $\pm$ 0.92
PRG in Carbopol 981	0.374 $\pm$ 0.06	0.59 $\pm$ 0.10	9.8 $\pm$ 1.61
PRG chitosan ME	0.233 $\pm$ 0.03	0.37 $\pm$ 0.04	6.1 $\pm$ 0.69
PRG sodium alginate ME	0.287 $\pm$ 0.07	0.45 $\pm$ 0.12	7.5 $\pm$ 1.93
PRG Carbopol 981 ME	0.288 $\pm$ 0.04	0.45 $\pm$ 0.06	7.6 $\pm$ 0.98

^aW/O/W MEs: water-in-oil-in-water microemulsions.

^bData are expressed as mean $\pm$ SEM; $n = 6$.

^cPRG: pregabalin. p value that represents the outcome of the one-way ANOVA analysis is 0.4191.

Pharmacodynamic Parameters after Topical Application of Single Dose of Different Pregabalin Formulations to Dutch Belted Rabbits ^a

Table 4.

pharmacodynamic parameters	ophthalmic eye drops				
	PRG ^b chitosan ME ^c	PRG sodium alginate ME	PRG Carbopol 981 ME	PRG in Carbopol 981	PRG W/O ME
baseline IOP ^d	21.2 ± 0.4	20.9 ± 0.2	21.4 ± 0.7	22.1 ± 0.4	16.7 ± 1.2
IOP at $T_{\max}$ ^e	13.9 ± 1.0	13.6 ± 0.2	12.3 ± 0.5	15.7 ± 0.4	12.7 ± 0.7
IOP	-7.3 ± 1.3	-7.3 ± 0.4	-9.1 ± 0.8	-6.5 ± 0.4	-4.0 ± 0.58
% reduction in IOP	34.1 ± 5.4	35.0 ± 1.7	42.3 ± 2.6	29.4 ± 1.4	23.8 ± 1.9
$T_{\max}$ (h)	4.0 ± 1.0	3.7 ± 0.3	3.3 ± 0.9	3.3 ± 1.5	2.33 ± 0.33
T_{end} ^f (h)	20.0 ± 0.0	24.0 ± 0.0	32.7 ± 1.3	9.3 ± 0.7	7.33 ± 1.33
AUC ^g (% . H)	323.0 ± 27.0	384.3 ± 21.2	788.6 ± 36.8	172.3 ± 16.9	98.9 ± 13.8

^a Data are expressed as mean ± SEM; $n = 3$.^b PRG: pregabalin.^c ME: microemulsion.^d IOP: intraocular pressure.^e $T_{\max}$: time to maximum response in hours.^f T_{end} (h): time to end of response in hours.^g AUC (% . H): total area under % IOP reduction *versus* time curve

Table 5. Statistical Comparisons of Pharmacodynamic Parameters after Topical Application of Single Dose of Different PRG Formulations

pharmacodynamic parameters	% reduction in IOP ^e	$T_{\max}$ ^d	T_{end} ^e	AUC ^f
Overall p value ^a	0.0818	0.473	<0.0001	<0.0001
Carbopol ME ^b vs Sod alginate ME	>0.05 ^g	>0.05	<0.001	<0.001
Carbopol ME vs Chitosan ME	>0.05	>0.05	<0.0001	<0.001
Carbopol ME vs Carbopol	>0.05	>0.05	<0.0001	<0.0001
Carbopol ME vs W/O ME	>0.05	>0.05	<0.0001	<0.0001
Sod alginate ME vs Chitosan ME	>0.05	>0.05	>0.05	>0.05
Sod alginate ME vs Carbopol	>0.05	>0.05	<0.0001	>0.05
Sod alginate ME vs W/O ME	>0.05	>0.05	<0.0001	<0.01
Chitosan ME vs Carbopol	>0.05	>0.05	<0.0001	>0.05
Chitosan ME vs W/O ME	>0.05	>0.05	<0.0001	<0.05
Carbopol vs W/O ME	>0.05	>0.05	>0.05	>0.05

^a Overall p value represents the outcome of the one-way ANOVA analysis.

^b ME: microemulsion.

^c IOP: intraocular pressure.

^d $T_{\max}$: time to maximum response in hours.

^e T_{end} : time to end of response in hours.

^f AUC: total area under % IOP reduction *versus* time curve.

^g Individual p values represent the outcome of Tukey's multiple comparisons test.