Novel Nanoparticulate Gel Formulations of Steroids for the Treatment of Macular Edema

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Abstract

Purpose: This article describes the development and characterization of PLGA nanoparticles of dexamethasone (DEX), hydrocortisone acetate (HA), and prednisolone acetate (PA) suspended in thermosensitive gels indicated for the treatment of macular edema (ME).

Methods: Nanoparticles were prepared by oil-in-water (O/W) emulsion and dialysis methods using PLGA 50:50 and PLGA 65:35. These particles were characterized for entrapment efficiency, size distribution, surface morphology, crystallinity, and *in vitro* release. Further, *ex vivo* permeation studies of DEX in suspension and nanoparticulate formulations were carried out across the rabbit sclera.

Results: Entrapment efficiencies of DEX, HA, and PA were found to be lower with the dialysis method. O/W emulsion/solvent evaporation technique resulted in higher entrapment efficiencies, that is, 77.3%, 91.3%, 92.3% for DEX, HA, and PA, respectively. Release from nanoparticles suspended in thermosensitive gels followed zero-order kinetics with no apparent burst effect. *Ex vivo* permeability studies further confirmed sustained release of DEX from nanoparticles suspended in thermosensitive gels.

Conclusions: These novel nanoparticulate systems containing particles suspended in thermosensitive gels may provide sustained retina/choroid delivery of steroids following episcleral administration.

Introduction

MACULAR EDEMA (ME) is caused by central extravascular swelling of the macula resulting in a significant loss of visual activity. The pathology is primarily associated with the leakage of fluids (from retinal blood vessels) and deposition of lipids and proteins beneath the center of the macula resulting in swelling. A more detailed study at the cellular level revealed that hypoxia causes thickening of the basement membrane of vascular endothelium and reduction in pericytes count that support the lining of retinal blood vessels. This thickening gradually spreads and distorts the central vision.^{1,2} ME is broadly classified into 2 types: cystoid macular edema (CME) and diabetic macular edema (DME). Ocular complications resulting from diabetes are the leading cause of adult blindness in the United States. Vision loss in diabetic patients is primarily associated with DME. Currently, ME is treated with laser photocoagulation and corticosteroids. Depending on the condition of a patient, laser treatment at different wavelengths may also be indicated in the treatment of ME.³ Laser treatment aids in suppressing further vision loss but may not repair lost

vision. Corticosteroids are well known as effective anti-inflammatory agents.⁴ Owing to their potent anti-inflammatory activity, these agents have gained considerable attention as therapeutic candidates for vision-threatening retinal diseases such as age-related macular degeneration, proliferative vitreoretinopathy, and DME.⁵ Treatment of the posterior segment requires localized drug delivery. In eye clinics, corticosteroids are widely applied through local (eye drops, ointments, implants, and intravitreal injections) and systemic routes (oral and parenteral routes).⁶ Drug administration by conventional routes fail to achieve required therapeutic concentrations in the eye due to the presence of ocular barriers.^{7,8} Though intravitreal injection directly delivers the compounds to the posterior segment of the eye, their inherent potential side effects like increased intraocular pressure,⁹ hemorrhage,¹⁰ retinal detachment,¹¹ cataract,¹² endophthalmitis,¹³ lead to complications limiting long-term therapy.¹⁴ Elimination therapy from the vitreous is governed by molecular weight and polarity. Intravitreal administration is found to be more suitable for high-molecular-weight (>500 Da) compounds displaying longer half-lives.¹⁵

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Steroid administration to the posterior segment through implants is a promising strategy in the treatment of DME. Two corticosteroid-based implants that include triamcinolone acetonide (I-vation™ TA, SurModics) and fluocinolone acetonide implant (Retisert®, Bausch & Lomb) are available in the market. Though implants overcome many of the disadvantages associated with intravitreal injections, surgical procedure, and risk of drug precipitation due to poor solubility may cause side effects.¹⁶ Recently, FDA has approved intravitreal dexamethasone (DEX) implant Ozurdex™ (administered via intravitreal injection) for the treatment of ME following branch or central retinal vein occlusion. However, it is associated with many adverse effects such as increased intraocular pressure, conjunctival hemorrhage, conjunctival hyperemia, cataract, ocular hypertension, and vitreous detachment (www.ozurdex.com).¹⁷

Subconjunctival drug administration is considered to be a localized noninvasive method and a suitable alternative to intravitreal injections/implants.¹⁸ A recent study demonstrated that subconjunctival injection of DEX disodium phosphate is more effective in delivering DEX into the subretinal fluid of patients compared to peribulbar injection or oral administration in patients suffering from rhegmatogenous retinal detachment.¹⁹ Hence, delivery of corticosteroids subconjunctivally may provide adequate therapeutic drug concentrations in the posterior segment.¹⁸ However, such treatments require frequent injections that need repeated clinical intervention. The other disadvantage of subconjunctival administration is dispersion of drug into anterior chamber. Conrad and colleagues conducted a study on the effect of instillation volume on the absorption pathways in albino rabbits following subconjunctival administration. They concluded that when injection volume was >200 µL most of the drug refluxed back into the anterior segment and absorption occurred via corneal route.²⁰ An ideal sustained drug delivery system for the treatment of DME should possess high entrapment efficiency, ability to deliver the drug in zero-order fashion, easy to manufacture, and a relatively noninvasive delivery route. Entrapment of steroids in biodegradable nanoparticulate systems may be an alternative strategy for long-term drug delivery to posterior segment. Polylactide (PLA) and polylactide-co-glycolide (PLGA) are the most widely used biodegradable polymers that are approved by FDA. These polymers are hydrolyzed to form natural metabolites (lactic and glycolic acids) that are eliminated from the body through Krebs' cycle.²¹ Nanoparticles can be further dispersed in PLGA-PEG-PLGA thermosensitive gels that may be conveniently injected subconjunctivally for sustained drug release.²² Moreover, thermogelling systems also help in preventing recirculation back into anterior segment thereby slowing down conjunctival clearance. These systems can form a depot above the sclera following subconjunctival injection and slowly release actives for the treatment of posterior segment diseases.

In this study, PLGA nanoparticles of steroids (DEX, HA, and PA) were prepared by dialysis and O/W emulsion/solvent evaporation methods. Nanoparticles were evaluated for entrapment efficiency, surface morphology, particle size, and *in vitro* release. Effect of lactide/glycolide ratio on the steroid release rate from the nanoparticles prepared by O/W emulsion/solvent evaporation method was investigated. Mechanism of drug release from the nanoparticles was delineated. We have also investigated the effect of PLGA-PEG-PLGA thermosensitive gel on the release mechanisms of steroids from PLGA

nanoparticles with optimum entrapment efficiency, drug loading, surface morphology, particle size, and *in vitro* release characteristics. Finally, permeation studies of DEX from suspension and nanoparticulate formulations were carried out across excised sclera to delineate *ex vivo* drug release pattern.

Methods

Materials

PLGA polymers, that is PLGA 50:50 (D,L-lactide:glycolide), molecular weight 45,000–75,000 Da, PLGA 65:35 (D,L-lactide:glycolide), molecular weight 45,000–75,000 Da, DEX, hydrocortisone acetate (HA), prednisolone acetate (PA), polyvinyl alcohol (PVA), tetrabutylammonium hydrogen sulfate (TBAHS), and polyethylene glycol 1450 were procured from Sigma Chemicals (St. Louis, MO). Hydroxyl propyl methyl cellulose (HPMC)—K4M premium grade was obtained from Dow Chemical Company. Dimethylformamide was obtained from American Scientific Products (Muskegon, MI). [³H]-Mannitol (specific activity: 50 mCi/mmol), a paracellular marker, was purchased from Amersham Biosciences, Ltd. (Piscataway, NJ). Thermosensitive gel PLGA-PEG-PLGA (weight average molecular weight [M_w]=4,759 Da) was synthesized and purified in our lab.

Development of PLGA-based nanoparticles

Dialysis method. PLGA-based nanoparticles were prepared by dialysis method without the incorporation of any surfactant. In brief, PLGA 50:50 and steroids (10:1 ratio by weight) were dissolved in 10 mL of dimethylformamide. The solution was introduced into a dialysis bag (MWCO=3,500 g/mol) and dialyzed against 1 L of distilled water with continuous stirring at room temperature for 24 h. Water was replaced after every 3 h. The resultant solution was centrifuged at 22,000g for 60 min. Nanoparticles formed were freeze-dried over 48 h.²³

O/W emulsion/solvent evaporation. PLGA nanoparticles containing DEX, HA, and PA were prepared by a single oil-in-water (O/W) emulsion/solvent evaporation method with a minor modification.²⁴ In order to maximize drug loading, PLGA (100 mg) was dissolved in dichloromethane (4 mL) and DEX or PA (16 mg) in 1 mL of acetone. DEX or PA in acetone was added to the PLGA in dichloromethane to form the organic phase. In a separate experiment, HA (16 mg) and PLGA (100 mg) were dissolved in 5 mL chloroform. Organic phase was slowly mixed with an aqueous solution containing 2.5%w/v PVA under continuous stirring.²⁵ An O/W type emulsion was formed upon sonication (Fisher 100 Sonic Dismembrator, Fisher Scientific) at a constant power output of 55 W for 5 min. The sample was kept in an ice bath during sonication to prevent any overheating of the emulsion. It was stirred gently at room temperature for 12 h. Subsequently, nanoparticle suspension was exposed to vacuum for 1 h to ensure complete removal of organic solvents. Untrapped drug and PVA residue were removed by washing nanoparticles 3 times with distilled water. The resultant solution was centrifuged at 22,000g for 60 min. Nanoparticles formed were freeze-dried over 48 h.

Entrapment efficiency of drug

For measuring drug entrapment in nanoparticles, 2 mg of freeze-dried sample was dissolved in 2 mL of

dichloromethane and mixed thoroughly for 24 h. After that these samples were then dried under inert atmosphere (nitrogen gas) and subsequently dissolved in 200 μ L of acetonitrile:water (70:30) and centrifuged at 12,000g for 10 min. The supernatant was aspirated for analysis of drug content by HPLC.²⁶ Entrapment efficiency and drug loading were calculated using Equations 1 and 2.

Entrapment efficiency (%)

$$= \frac{(\text{Amount of drug remained in nanoparticles})}{(\text{Initial drug amount})} \times 100 \quad \text{Eq. 1}$$

Drug loading (%)

$$= \frac{(\text{Weight of drug in nanoparticles})}{(\text{Weight of nanoparticles})} \times 100 \quad \text{Eq. 2}$$

In vitro drug release

Drug-loaded nanoparticles (5 mg) were dispersed in 1 mL isotonic phosphate-buffered saline (IPBS), pH 7.4 and subsequently introduced into dialysis bags (MWCO—6,275 g/mol). PLGA 65:35 nanoparticles containing DEX, HA, or PA were suspended in 1 mL of 23% w/w PLGA-PEG-PLGA aqueous polymer solution and then added to dialysis bags. The polymer solution inside the bags formed gels at 37°C with exposure times over 30–60 s.²⁷ The dialysis bags were introduced into vials containing 10 mL IPBS and 0.025% w/v sodium azide to avoid microbial growth and 0.02% (w/v) Tween 80 to maintain sink condition.^{28,29} The vials were placed in a shaker bath at 37 $\pm$ 0.5°C and 60 oscillations/min. At regular time intervals, 200 μ L of samples were withdrawn and replaced with equal volumes of fresh buffer. Samples were analyzed by HPLC. Experiments were conducted in triplicates.

HPLC analysis

High-performance liquid chromatography system (Waters 600 pump; Waters, Milford, MA), equipped with a UV detector (RAININ, Dynamax, Absorbance Detector Model UV-C) and reversed-phase C8 column (5 μ m, 100A; Microsorb, Woburn, MA), was employed. All samples were analyzed by isocratic method with a mobile phase containing 10 mM TBAHS (pH 3.0) and 30% acetonitrile pumped at a flow rate of 1 mL/min. The detector was set at a wavelength of 254 nm. The retention times of DEX, HA, and PA were 16.2, 26.2, and 24.6 min, respectively. Validation parameters are shown in Table 1.

Drug release mechanism

Depending on the drug release pattern, various kinetic models (zero-order equation or Higuchi equation) were

employed in the calculation of release rate constants. In Higuchi equation (Eq. 3), cumulative percentage of drug released was plotted against square root of time.

$$Q = K t^{1/2} \quad \text{Eq. 3}$$

K is the apparent first-order release rate constant and t represents time in hours. K values are calculated from data representing <60% release.³⁰ Zero-order rate equation (Eq. 4) was employed for calculating the release rate constants from gel formulations.

$$Q = K_0 t \quad \text{Eq. 4}$$

K_0 is the apparent zero-order release rate constant and t represents time in hours.

Surface morphology

Scanning electron microscopy (FEG ESEM XL 30, FEI, Hillsboro, OR) was employed for studying surface morphology. Freeze-dried nanoparticles were attached to a double-sided tape, spray-coated with gold/palladium at 0.6 kV, and then examined under the electron microscope.

Particle size

A dynamic light scattering (Brookhaven Zeta Plus instrument, Holtsville, NY) technique was employed to measure the particle size and polydispersity values in triplicates.

Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) curves were obtained by a Thermal Analysis Instruments-DSC-60, Shimadzu Corporation. Samples (10–15 mg) were loaded in aluminum crucibles and subjected to a heating cycle from 25°C to 350°C with a heating rate of 10°C/min. A steady stream of nitrogen gas was used for controlling the heating and cooling rates. Polymer samples, pure drugs, and physical mixtures served as controls.

Storage stability studies

Lyophilized formulations of FDEX 65:35, FHA 65:35, and FPA 65:35 were sealed in ampoules and stored at 4°C. At regular time intervals, samples were removed and analyzed for gelation behavior and entrapment efficiency.

Tissue preparation

Adult New Zealand male rabbits weighing between 2 and 2.5 kg were obtained from Myrtle's Rabbitry (Thompson Station, TN). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of University of Missouri- Kansas City (UMKC, Kansas City, MO). Rabbits were anesthetized with intramuscular

TABLE 1. VALIDATION PARAMETERS FOR THE HPLC METHOD OF ANALYZING STEROIDS

Drug	Linearity			LOQ ng/mL	LOD ng/mL	Range μ g/mL	Repeatability (%)	Accuracy (%)
	Slope	Constant	R^2					
DEX	166197	193671	0.9994	700	200	0.78–6.25	1.68–6.60	95.50–98.90
HA	201599	–22003	0.9991	750	250	0.78–6.25	1.42–4.21	96.80–102.31
PA	157138	355428	0.9951	750	250	0.78–6.25	2.11–3.80	98.31–103.65

Abbreviations: DEX, dexamethasone; HA, hydrocortisone acetate; HPLC, high-performance liquid chromatography; PA, prednisolone acetate.

TABLE 2. ENTRAPMENT EFFICIENCY OF NANOPARTICLES PREPARED BY DIALYSIS METHOD AND O/W EMULSION/SOLVENT EVAPORATION METHOD

Drug	Dialysis method		O/W emulsion/solvent evaporation method			
	PLGA 50:50		PLGA 50:50		PLGA 65:35	
	(mean $\pm$ SEM) %	DL (%)	(mean $\pm$ SEM) %	DL (%)	(mean $\pm$ SEM) %	DL (%)
DEX	7.2 $\pm$ 3.5	0.71 $\pm$ 0.11	77.3 $\pm$ 4.4	10.1 $\pm$ 1.1	76.6 $\pm$ 3.1	9.9 $\pm$ 2.2
HA	12.1 $\pm$ 2.7	1.19 $\pm$ 0.39	91.3 $\pm$ 3.5	11.2 $\pm$ 1.5	92.3 $\pm$ 2.9	12.1 $\pm$ 1.4
PA	14.3 $\pm$ 1.3	1.40 $\pm$ 0.24	92.3 $\pm$ 5.1	10.9 $\pm$ 1.2	94.3 $\pm$ 3.3	11.1 $\pm$ 0.9

Abbreviations: DEX, dexamethasone; DL, drug loading; HA, hydrocortisone acetate; O/W, oil-in-water; PA, prednisolone acetate; PLGA, polylactide-co-glycolide; SEM, standard error of mean.

administration of ketamine HCl (35 mg/kg) and xylazine (5 mg/kg). Animals were euthanized by an overdose of sodium pentobarbital (100 mg/kg) administered through marginal ear vein under deep anesthesia. Eyes were removed carefully, then excised, and the posterior segment was separated by cutting along the corneal/scleral limbus junction. Tissue segments were separated and the sclera was placed in a Petri dish containing Dulbecco's phosphate-buffered saline. The superotemporal region of each globe was retained, avoiding the vortex veins, the anterior ciliary perforating vessels, and tissue closer than 2 mm to the optic nerve.

Permeability studies

The excised tissue was mounted on a Franz-type vertical diffusion cell (PermeGear Inc., Hellertown, PA) for carrying out permeability studies. The episcleral side was placed toward the donor chamber on which the drug depot was placed. The receptor chamber was filled with IPBS containing sodium azide. An aliquot (300 μ L) was withdrawn at regular time intervals and replaced with equal amounts of fresh buffer. All the experiments were carried under sink conditions. Samples were analyzed by HPLC as described earlier. Histological examination and [^{3} H]-mannitol permeability studies across sclera were carried out to observe the basic changes in the anatomy of the sclera tissues. [^{3} H]-Mannitol permeability samples were transferred into scintillation vials, mixed with 5 mL scintillation cocktail, and analyzed for radioactivity. All the permeability studies were carried out in triplicates.

Permeability (P_{app}) of the drug from suspension and formulations was calculated using Equation 5.

$$\text{Permeability } (P_{app}) = \text{Flux}/C_d \quad \text{Eq. 5}$$

Flux (J) is calculated by dividing the slope obtained by plotting cumulative amount of drug permeated (M) through the sclera vs. time (t) with cross-sectional area of the membrane (A) exposed to the drug. C_d is the initial drug concentration in the donor chamber.

Results

Emulsion/solvent (O/W) evaporation method resulted in higher steroid entrapment efficiencies compared to the dialysis method. Nanoparticle entrapment efficiencies for DEX, HA, and PA by dialysis method with PLGA 50:50 were found to be low (5%–15%). Table 2 represents the experimental values of entrapment efficiencies and drug loading. Though steroids are fairly lipophilic ($\log P$ values between 1.6 and 1.8), entrapment efficiency and drug loading values were unexpectedly low (Table 2). Dialysis method resulted in various sizes ranging between 200 and 250 nm with unimodal size distribution and fairly high polydispersity values (Table 3). Further, these particles exhibited a biphasic release pattern and could not sustain the release of DEX, HA, and PA over extended time periods. DEX was completely released from nanoparticles within 48 h. In case of HA and PA, the release was observed over 96 h. Release data of DEX, HA, and PA were plotted according to Higuchi equation for estimating the release rate constants that were found to be 16.0, 5.4, and 7.10 $\text{h}^{-1/2}$ for DEX, HA, and PA, respectively (Table 4). Release profiles of DEX, HA, and PA are illustrated in Figure 1. Drug release data was fitted into Korsmeyer-Peppas equation (data not shown). A better linear regression coefficient was obtained by plotting the release data according to Higuchi model (Eq. 3) relative to Korsmeyer-Peppas model. O/W emulsion/solvent evaporation method resulted in higher

TABLE 3. PARTICLE SIZE AND POLYDISPERSITY VALUES OF NANOPARTICLES PREPARED BY DIALYSIS METHOD AND O/W EMULSION/SOLVENT EVAPORATION METHOD

Drug	Dialysis method		O/W emulsion/solvent evaporation method			
	PLGA 50:50		PLGA 50:50		PLGA 65:35	
	Particle size (nm)		Particle size (nm)		Particle size (nm)	
	mean $\pm$ SEM	Polydispersity	mean $\pm$ SEM	Polydispersity	mean $\pm$ SEM	Polydispersity
DEX	216 $\pm$ 4	0.10	204 $\pm$ 4	0.057	203 $\pm$ 3	0.033
HA	251 $\pm$ 3	0.09	226 $\pm$ 3	0.040	214 $\pm$ 2	0.019
PA	237 $\pm$ 6	0.12	183 $\pm$ 1	0.047	193 $\pm$ 2	0.005

Abbreviations: DEX, dexamethasone; DL, drug loading; HA, hydrocortisone acetate; O/W, oil-in-water; PA, prednisolone acetate; PLGA, polylactide-co-glycolide; SEM, standard error of mean.

TABLE 4. RELEASE RATE CONSTANTS OF DRUGS FROM NANOPARTICLES PREPARED BY DIALYSIS METHOD, O/W EMULSION/SOLVENT EVAPORATION METHOD, AND FORMULATIONS

Drug	Dialysis method		O/W emulsion/solvent evaporation method				Formulations (PLGA 65:35 nanoparticles in thermosensitive gels)	
	PLGA 50:50		PLGA 50:50		PLGA 65:35		PLGA 65:35	
	$Q = K t^{1/2}$		$Q = K t^{1/2}$		$Q = K t^{1/2}$		$Q = K_0 t$	
	$K (h^{-1/2})$	R^2	$K (h^{-1/2})$	R^2	$K (h^{-1/2})$	R^2	$K (\mu g/h)$	R^2
	mean $\pm$ SEM		mean $\pm$ SEM		mean $\pm$ SEM		mean $\pm$ SEM	
DEX	16.0 $\pm$ 0.40	0.93	6.73 $\pm$ 0.30	0.95	5.11 $\pm$ 0.12	0.97	0.24 $\pm$ 0.01	0.92
HA	5.40 $\pm$ 0.30	0.93	2.24 $\pm$ 0.18	0.98	1.31 $\pm$ 0.20	0.96	0.21 $\pm$ 0.04	0.95
PA	7.10 $\pm$ 0.60	0.92	3.40 $\pm$ 0.20	0.95	3.16 $\pm$ 0.14	0.97	0.23 $\pm$ 0.02	0.97

Abbreviations: DEX, dexamethasone; DL, drug loading; HA, hydrocortisone acetate; O/W, oil-in-water; PA, prednisolone acetate; PLGA, polylactide-co-glycolide; SEM, standard error of mean.

entrapment efficiency ranging between 70% and 95% for DEX, HA, and PA. PVA was added as a stabilizing agent in the preparation of nanoparticles as it is known to considerably reduce the size of nanoparticles.²⁵ O/W emulsion/solvent evaporation method resulted in nanoparticles with unimodal size distribution (Fig. 2). The mean particle size of steroid nanoparticles (~200 nm) and polydispersity values are shown in Table 3. Entrapment efficiency and particle size did not change appreciably with lactide/glycolide ratio (PLGA 50:50 and PLGA 65:35) in the polymers. The polydispersity values were found to be close to zero. Emulsion/solvent (O/W) evaporation method resulted in uniform size particles with significant increase in entrapment efficiency and drug loading as compared to the nanoparticles prepared by dialysis method. So, further characterization studies were carried out for the nanoparticles prepared by O/W emulsion/solvent evaporation method. Scanning electron microscopy pictures (Fig. 3) confirm the size uniformity and spherical shape of the particles with a smooth surface texture. Irrespective of the lactide/glycolide ratio, a biphasic release pattern consisting of an initial rapid phase (burst) followed by sustained release was observed from the nanoparticles. Release data of DEX, HA, and PA was plotted according to Higuchi equation. The release rate constants of

DEX, HA, and PA were found to be 6.73, 2.24, and 3.40 $h^{-1/2}$, respectively. Higher lactide/glycolide ratio in PLGA altered the release pattern of steroids. Initial burst release of DEX, HA, and PA was found to be less in nanoparticles prepared from PLGA 65:35. Release rate constants were found to be 5.11, 1.31, and 3.16 $h^{-1/2}$ for DEX, HA, and PA, respectively, from PLGA 65:35 nanoparticles (Table 4). Release profiles of DEX, HA, and PA are illustrated in Figure 4. Raising the lactide/glycolide ratio further prolonged the release of steroids from nanoparticles.²⁸ This effect may be due to the increase in polymer hydrophobicity that in turn retards the rate of water penetration into polymer matrices. The release rate constants along with regression values are shown in Table 4. Based on the *in vitro* release data, we found that PLGA 65:35 is superior to PLGA 50:50 for the preparation of steroid nanoparticles. Hence, we proceeded further with the characterization of PLGA 65:35 nanoparticles. DSC studies were conducted to determine crystalline/amorphous nature of the entrapped drug inside the PLGA 65:35 nanoparticles.³¹ PLGA 65:35 exhibited a glass transition temperature at 49.78°C (Fig. 5A). DEX, HA, and PA (Fig. 5B–5D) exhibited a sharp endothermic peak corresponding to the melting points (263.14°C, 224.36°C, and 241.9°C) possibly indicating their crystalline nature. Endothermic peaks of both polymer and drug in the physical mixtures (Fig. 5E–5G) are clearly evident. Melting peak of drugs completely disappeared in nanoparticles of HA and PA (Fig. 5I and 5J), while nanoparticles of DEX exhibited slight melting point peak (Fig. 5H) evident at 257.44°C. Profiles of DEX, HA, and PA release from the formulations prepared by dispersing PLGA 65:35 nanoparticles in PLGA-PEG-PLGA thermosensitive gel are also obtained. Synthesis and characterization of triblock copolymer PLGA-PEG-PLGA (weight average molecular weight [M_{wb}] determined by gel permeation chromatography—4,759 Da) have already been published from our laboratory.²⁷ Phase transition studies revealed the polymer concentrations ranging between 20% and 25% w/v forms gel at 32°C–60°C.²⁷ As the temperature inside the eye ranges from 34°C to 37°C, such polymeric gels may be appropriate for delivery.³² Burst release of active ingredients has been considerably retarded when nanoparticles are dispersed in thermosensitive gels. Moreover, a clear zero-order release pattern of steroids was observed from these formulations (Fig. 4). Storage stability studies were carried out for FDEX 65:35, FHA 65:35, and FPA 65:35 at 4°C. No significant change in the physical appearance and entrapment efficiency was observed after 3 months

FIG. 1. Percent cumulative release of dexamethasone (DEX), hydrocortisone acetate (HA), and prednisolone acetate (PA) from polylactide-co-glycolide (PLGA) 50:50 nanoparticles prepared by dialysis method ($n = 3$). Error bars represent the standard error of mean.

FIG. 2. Particle size distribution curves of nanoparticles prepared by oil-in-water (O/W) emulsion/solvent evaporation method. (A) dexamethasone (DEX), 50:50, (B) DEX 65:35, (C) hydrocortisone acetate (HA) 50:50, (D) HA 65:35, (E), prednisolone acetate (PA) 50:50, and (F) PA 65:35.

storage. Gelation property of the formulations was tested by tube inversion method at 34°C–37°C. All the formulations exhibited gelation in <60 s (Table 5, Fig. 6). *Ex vivo* permeability studies of DEX were carried out across excised rabbit sclera. Suspension of DEX (containing 0.5% w/v HPMC), DEX 65:35 nanoparticles, and FDEX 65:35 (nanoparticles of DEX prepared by PLGA 65:35 dispersed in PLGA-PEG-PLGA thermosensitive gels) were employed for permeation studies. Histological sections of sclera were examined by hematoxylin/eosin stain. No change in integrity, density, and interlacing of fibers was observed after 1, 2, and 4 days. The nuclei of fibroblasts were clearly visible even after 4 days of experimentation (Fig. 7). The membrane integrity of sclera was further confirmed by carrying out [^{3} H]-mannitol permeability studies. Permeability values (mean $\pm$ SEM) of [^{3} H]-mannitol on days 1 and 4 were found to be $1.47\text{E-}05 \pm 2.52\text{E-}06$ and $2.01\text{E-}05 \pm 6.44\text{E-}07$ cm/s, respectively. No significant difference in the permeability of [^{3} H]-mannitol was evident. Permeability values ($P \times 10^6$) of DEX from

suspension, DEX 65:35 nanoparticles, and FDEX 65:35 were found to be 2.23, 0.40, and 0.11 cm/s, respectively (Fig. 8, Table 6). Cumulative amount of DEX permeated across the sclera from suspension, DEX 65:35 nanoparticles, and FDEX 65:35 were found to be 5.94, 0.85, and 0.28 $\mu\text{g/h}$, respectively (Table 6).

Discussion

Treatment of severe DME infections require sustained delivery of steroids to the posterior segment. Delivery of high corticosteroid doses intravitreally has limitations due to side effects such as endophthalmitis, retinal detachment, and other undesirable events related to repeated injections. Subconjunctival deposition is a promising approach for the drug delivery to retina/choroid. Incorporation in nanoparticles will protect these anti-inflammatory agents from enzymatic degradation and will provide sustained release that may in turn reduce the dosing frequency.¹⁷ Weijten

FIG. 3. Scanning electron microscopy pictures of nanoparticles prepared by oil-in-water (O/W) emulsion/solvent evaporation method. (A) dexamethasone (DEX) 50:50, (B) DEX 65:35, (C) hydrocortisone acetate (HA) 50:50, (D) HA 65:35, (E) prednisolone acetate (PA) 50:50, and (F) PA 65:35.

and coauthors³³ concluded that DEX tissue concentrations following frequent topical dosing in various ocular tissues is far lower than subconjunctival injection. Sustained drug delivery to the posterior segment can be achieved by subconjunctival administration of steroid nanoparticles in ME patients.¹⁸ Moreover, drugs administered subconjunctivally easily reach retinal pigment epithelium because sclera and choroid offer very limited resistance to drug transport.³⁴ In the present study, PLGA nanoparticles of DEX, HA, and PA were prepared, characterized, and tested for the *ex vivo* drug release profiles in excised rabbit sclera. Such delivery systems may generate therapeutic levels from low doses of DEX, HA, and PA at the retina/choroid reducing inflammation and toxicity. Moreover, drug encapsulation in polymer matrices may enhance drug stability and reduce toxicity.

PLGA nanoparticles belong to colloidal drug delivery systems with size ranging from 10 to 1,000 nm. With a mean diameter of 200 nm, these particles are considered to be optimal for subconjunctival delivery as studies carried out by Amrite and colleagues revealed that 200 nm and higher polystyrene particles (carboxylate modified, negatively charged) retain for longer durations following subconjunctival administration.²² Moreover, higher size particles may result in occlusion of 30G needle used for subconjunctival injection. Unlike the non-biodegradable polystyrene

particles, PLGA polymer used in our study is biodegradable in nature and known to exhibit burst release of drugs. Our main intension is to prevent the burst release of steroids by dispersing the PLGA nanoparticles in thermosensitive gels. These formulations may provide sustained retina/choroid delivery of steroids following episcleral administration.

As steroids are fairly lipophilic ($\log P$ values between 1.6 and 1.8) dialysis method was employed in the preparation of PLGA nanoparticles. However, this method resulted in nanoparticles with poor encapsulation efficiency and could not sustain the drug release over longer durations. In dialysis method, nanoparticles are formed by continuous precipitation of polymer and drug inside the dialysis bag with the removal of organic solvent. The rate of precipitation depends on the relative lipophilicities of polymer and drug. Since PLGA polymer is highly lipophilic and practically insoluble in water, it precipitates at a faster rate relative to drug. As a result, drug molecules are adsorbed on to the surface of the polymer rather than being entrapped inside the core of the particles. Moreover, dialysis is carried out for 24 h, which may result in release of the adsorbed/entrapped drug into the aqueous phase. In contrast, O/W emulsion/solvent evaporation method proved to be superior to the dialysis method in terms of entrapment efficiency and uniformity. O/W emulsion/solvent evaporation method resulted in consistently low entrapment values of DEX as compared to HA and PA. This may be attributed to the higher affinity of DEX to external aqueous phase as compared to HA and PA resulting in lower drug content inside the matrix of nanoparticles. Uniform particle sizes were obtained with mean effective diameters of 180–230 nm and polydispersity of 0.005–0.057. Size of nanoparticles depends on the various formulation parameters such as volume of the aqueous phase, drug, and polymer concentration in the organic phase and concentration of the surfactant in the aqueous phase.³⁵ Kim and colleagues reported the formation of DEX nanoparticles with size ranging between 400 and 600 nm following the use of dichloromethane:acetone ratio of 1:1 and a polymer concentration of 27 mg/mL in organic phase.²⁴ In the present study, we have added dichloromethane:acetone in a ratio of 4:1 and maintained a polymer concentration of 20 mg/mL in organic phase. This solvent composition significantly reduced the particle size to 180–230 nm with more uniform distribution. This result can be attributed to the decrease in organic phase viscosity that in turn leads to a net increase in shear stress experienced by the organic phase. Moreover, the slow and steady diffusion of acetone into external aqueous phase leads to the formation of smaller and more uniform size particles. Use of chloroform in the preparation of HA nanoparticles slightly increased the particles size irrespective of the polymer used. Except for the duration, the release patterns of HA and PA was similar from PLGA 50:50 and PLGA 65:35 matrices. DEX nanoparticles prepared with PLGA 65:35 released the cargo in a sustained and controlled fashion (Fig. 4A). This may be due to the crystalline arrangement of DEX inside the nanoparticles that is also evident from the DSC results (Fig. 5H). DSC studies of PLGA 65:35 nanoparticles indicate the presence of drug particles (HA and PA) either in amorphous or disordered crystalline state.³⁶

DSC thermograms depict the phase transition temperatures (T_g or T_m) of polymer and drugs either in pure form or in combination with polymer. Drug and polymer matrix may exist in 4 different forms: (a) amorphous drug in crystalline

FIG. 4. Percent cumulative release profiles of steroids from nanoparticles prepared by oil-in-water (O/W) emulsion/solvent evaporation method ($n = 3$) and nanoparticles suspended in thermosensitive gels. (A) dexamethasone (DEX), from polylactide-co-glycolide (PLGA) 50:50, PLGA 65:35 nanoparticles and formulation (DEX-PLGA 65:35 suspended in PLGA-PEG-PLGA gels), (B) HA from PLGA 50:50, PLGA 65:35 nanoparticles and formulation (HA-PLGA 65:35 suspended in PLGA-PEG-PLGA gels), and (C) PA from PLGA 50:50, PLGA 65:35 nanoparticles and formulation (PA-PLGA 65:35 suspended in PLGA-PEG-PLGA gels). Error bars represent the standard error of mean.

polymer, (b) amorphous drug in amorphous polymer, (c) crystalline drug in amorphous polymer, and (d) crystalline drug in crystalline polymer.³¹ DSC thermogram also aids in delineation of abrupt changes in the drug/polymer matrix due to specific drug polymer interactions. DEX, HA, and PA exhibited a sharp endothermic peak corresponding to the melting points indicating their crystalline nature. Melting peak of drugs completely disappeared in nanoparticles of HA and PA suggesting that the drug particles may be in amorphous or disordered crystalline form, while nanoparticles of DEX exhibited some crystallinity that was indicated by the slight melting point peak (Fig. 5H) evident at 257.44°C.³⁷ Burst

release phase from PLGA nanoparticles may be attributed to the presence of the active ingredients in amorphous state. Water can penetrate more easily through the amorphous/disordered crystalline matrices as compared to crystalline matrices initiating burst release.³⁸ Such effect is completely eliminated when nanoparticles are dispersed in thermosensitive gels. This may be due to the polymer adhesion to nanoparticle suspended in thermosensitive gels. Such nanoparticulate formulations may provide sustained release of steroids following subconjunctival administration. Previous studies from our laboratory indicated that the rate of drug release from microparticles *in vivo* is significantly slower

than *in vitro* conditions. This effect may be due to more rapid and continuous agitation as well as faster penetration of the solvent.³⁹ Moreover nanoparticles can be suitably formulated into the thermosensitive gels that are liquid at room temperature and can be administered subconjunctivally. The gel aids in controlling the burst release of drugs from the nanoparticles and also prevents dispersion of nanoparticles into anterior chamber after subconjunctival injection. The formulations can also help in the formation of a depot at the site of administration providing a robust concentration

gradient for rapid and efficient permeation of drugs across the sclera in a sustained manner.²² No significant change in the entrapment efficiency of steroids was observed after 3 months storage at 4°C. All formulations exhibited spontaneous gelation with no visible clumping of nanoparticles. This indicates the uniform nanoparticle dispersion inside thermosensitive gel. Posurdex™ (Allergan) is a biodegradable implant of DEX that contains either 350 or 700 µg dose in PLGA copolymer. This system has undergone phase II clinical trial in patients suffering from DME, post cataract surgery, and uveitis. Promising results were obtained from the implants containing 700 µg DEX. Significant improvement in the vision was obtained from the implants as compared to the control group. Moreover, the drug from these implants is released over the duration of 5 weeks.⁴⁰ In the present study, nanoparticles containing DEX equivalent to 700 µg were used for carrying out transcleral studies. Samples were collected from the receptor chamber at regular time intervals and sink conditions were maintained throughout the experiment. Permeability studies were carried out until 5% of the donor drug concentration appeared in the receptor chamber. This approach avoids the preconceived notion of back diffusion of drug and also helps to maintain constant steady-state concentration gradient. Permeation of DEX from suspension containing 0.5% HPMC occurred for nearly 4 h (ie, 25–35 µg of DEX permeated the sclera in 4 h). DEX 65:35 nanoparticles on the other hand sustained drug release for 40 h. Further prolongation in the duration of release was observed when nanoparticles were suspended in thermosensitive gels. Permeability values of DEX significantly decreased when drug was entrapped in the nanoparticles and suspended in thermosensitive gels. The release of DEX from these formulations may act as the major rate-limiting step for the transcleral permeation. Significant difference in the duration of release was observed from FDEX 65:35 under *in vitro* and *ex vivo* conditions. This result may be attributed to the surface area of suspended particles (suspended in dialysis) that is exposed to the release medium, continuous motion of shaker bath, and nature of dialysis bag membrane. However, *in vitro* release conditions may not be applicable

FIG. 5. DSC thermograms of (A) PLGA 65:35, (B) DEX, (C) HA, (D) PA, (E) physical mixture of DEX + PLGA 65:35, (F) physical mixture of HA + PLGA 65:35, (G) physical mixture of PA + PLGA 65:35, (H) DEX 65:35 nanoparticles, (I) HA 65:35 nanoparticles, and (J) PA 65:35 nanoparticles. Abbreviations: DEX, dexamethasone; HA, hydrocortisone acetate; PA, prednisolone acetate; PLGA, polylactide-co-glycolide.

FIG. 6. Gelation and uniform particle dispersion of FDEX 65:35 after 3 months storage at 4°C. (A) FDEX 65:35 in solution at 25°C and (B) FDEX 65:35 in gel form at 34°C–37°C.

FIG. 7. Histological sections of scleral samples stained by hematoxylin/eosin dye. (A) Control, (B) after 1 day, (C) after 2 days, and (D) after 4 days. Bar represents 50 μ M. The black color dots represent the nuclei of the scleral fibroblast cells.

in the actual *in vivo* environment (following subconjunctival injection). Transcleral permeability studies simulate the *in vivo* conditions to a large extent provided proper sink conditions are maintained. Histological examination of sclera samples did not exhibit any noticeable changes after the completion of permeability studies. This observation was further confirmed by transport of [^{3} H]-mannitol across the sclera. As >60% of the sclera is made of water channels, [^{3} H]-mannitol can be used as a good marker for membrane integrity.³² No significant difference in [^{3} H]-mannitol transport was observed that confirms tissue integrity of sclera.

In target tissues such as retina/choroid/vitreous, DEX concentrations ranging from 10 to 4,000 ng/mL are required for effective treatment of various inflammatory conditions.⁴¹ From the *in vitro* release studies, we could observe a clear zero-order release pattern of steroids from the formulations. Further, from the *ex vivo* permeability data the cumulative

TABLE 5. STORAGE STABILITY DATA OF FDEX 65:35, FHA 65:35, AND FPA 65:35 AT 4°C

Samples	Time			
	0 month		3 months	
	Encapsulation efficiency (%)	Gelation time (s)	Encapsulation efficiency (%)	Gelation time (s)
FDEX 65:35	75.3 $\pm$ 1.9	30–60	76.1 $\pm$ 2.1	30–60
FHA 65:35	93.1 $\pm$ 2.1	30–60	92.3 $\pm$ 1.9	30–60
FPA 65:35	93.4 $\pm$ 2.3	30–60	94.5 $\pm$ 1.9	30–60

amount of DEX permeated across the sclera from nanoparticles suspended in thermosensitive gels was found to be 0.28 μ g/h. Using the formulations described in this article, DEX concentrations can be maintained well above the minimum effective concentrations for duration of several months following single episcleral administration. Depending on the severity of inflammation, various combinations of nanoparticles and thermosensitive gels (with varying molecular weights) loaded with steroids can be tailor-made so as to match the particular dosing requirements of a particular patient. Moreover, these nanoparticulate formulations may sustain the duration of drug release for a longer time period than Posurdex^{TM} (duration of drug release only 5 weeks). Hence, these formulations have an edge as compared to the implantable systems both in terms of duration of drug release and mode of administration.

Conclusions

In summary, we have provided proof for a novel injectable and controlled-release formulations of DEX, HA, and PA that can be used in the treatment of ME. PLGA 65:35 nanoparticles are found to be superior in terms of polydispersity and drug release profile. Burst release of drug from nanoparticles may be successfully controlled and/or eliminated by suspending in PLGA-PEG-PLGA thermosensitive gels. Moreover, entrapment inside the gel structure allows nanoparticle retention at the site of administration, thereby releasing the drug in a continuous manner. Histological studies and [^{3} H]-mannitol data confirmed that the integrity

FIG. 8. Permeability of dexamethasone (DEX) from suspension, DEX 65:35 nanoparticles, and FDEX 65:35 (DEX 65:35 nanoparticles suspended in thermosensitive gels). Inset: Permeability of DEX from suspension containing 0.5% w/v hydroxypropyl methylcellulose (HPMC). Error bars represent the standard error of mean.

TABLE 6. *Ex Vivo* PERMEABILITY OF DEX FROM SUSPENSION, DEX 65:35 NANOPARTICLES, AND FDEX 65:35 (DEX 65:35 NANOPARTICLES SUSPENDED IN THERMOSENSITIVE GELS)

S. No	Samples	Cumulative amount (μg) of DEX permeated per hour (mean $\pm$ SEM)	Permeability (cm/s) $\times 10^6$ (mean $\pm$ SEM)	R^2
1	DEX suspension	5.94 ± 0.50	2.23 ± 0.12	0.97
2	DEX 65:35 nanoparticles	0.85 ± 0.05	0.40 ± 0.08	0.97
3	FDEX 65:35	0.28 ± 0.03	0.11 ± 0.02	0.96

Abbreviation: SEM, standard error of mean.

of sclera was not compromised with these thermosensitive gel formulations. Following complete drug release from formulations, the remaining nanoparticulate shells degrade inside the body obviating the need for surgical removal as required for implants. *In vivo* pharmacokinetic studies should be carried out to determine the choroidal/retinal/vitreous concentrations following subconjunctival administration. Novel formulation strategies of nanoparticles suspended in thermosensitive gels can serve as an alternative to the current therapies like implants and intravitreal injections.

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Author Disclosure Statement

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