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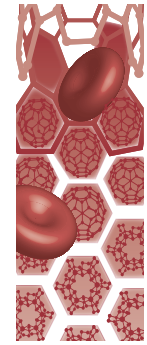

Pentablock copolymer dexamethasone nanoformulations elevate MYOC: *in vitro* liberation, activity and safety in human trabecular meshwork cells

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Aim: The aim of this study is to examine the elevation of MYOC in long-term treatment of human trabecular meshwork (HTM) cells using dexamethasone (DEX) encapsulated pentablock (PB) copolymer-based nanoparticles (NPs) (DEX-PB-NPs). **Materials & methods:** PB copolymers and DEX-PB-NPs were synthesized and characterized using nuclear magnetic resonance, gel permeation chromatography, and X-ray diffraction analyses. MYOC levels secreted from HTM cells were measured by western blot (WB) analysis. **Results:** DEX-PB-NPs were formulated in the size range of 109 ± 3.77 nm (n = 3). A long term DEX release from the NPs was observed over three months. Cell viability and cytotoxicity were not affected up to 12 weeks of treatment with PB-copolymer or DEX-PB-NPs. WB data from five HTM cell strains showed that MYOC levels increased by 5.2 ± 1.3, 7.4 ± 4.3, and 2.8 ± 1.1-fold in the presence of DEX-PB-NPs compared with 9.2 ± 3.8, 2.2 ± 0.5, and 1.5 ± 0.3-fold at 4, 8 and 12 weeks in control-DEX treatment group, respectively (n = 5). Based on the decline in MYOC levels after withdrawal of DEX from control wells, DEX-PB-NPs released the DEX for at least 10 weeks. **Conclusion:** The treatment of HTM cells using DEX-PB-NPs were analyzed in this study. The *in vitro* cell-based system developed here is a valuable tool for determining the safety and effects of steroids released from polymeric NPs.

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Keywords: block copolymer • MYOC • nanoparticle • steroids • sustained release • trabecular meshwork cells • western blot

Nanotechnology has opened exciting therapeutic options in drug delivery approaches. These systems offer several benefits in ocular drug delivery due to their small size and use of biodegradable materials in their formulation [1,2]. Dexamethasone (DEX), one of the most effective corticosteroids, has been widely indicated in the clinical practice of ophthalmology as an anti-inflammatory and immunosuppressive agent. It can be administered via topical, periocular (i.e., subconjunctival) or intraocular (i.e., intravitreal) routes. Topical DEX has proven efficacious in the management of postoperative inflammation in the anterior segment after cataract surgery, treatment of anterior uveitis (iritis) and dry-eye disease symptoms [3–5]. Intravitreal administration of DEX has been effective in the treatment of macular edema following retinal vein occlusion, diabetic macular edema [6–8], and non-infectious uveitis [9], particularly when other therapeutic agents have failed to provide treatment benefits. However, DEX has a short half-life [10], and requires multiple applications. As a consequence, technologies that achieve slow, sustained, and controlled-release of DEX may prevent frequent administrations or multiple invasive treatments [11,12]. In this regard, US FDA approved biodegradable polymers such as polycaprolactone (PCL), polylactic acid (PLA), polyglycolic acid

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(PGA) polyethylene glycol (PEG) and polylactide-co-glycolide (PLGA) have been comprehensively studied for the sustained delivery of the corticosteroid [13,14]. These polymers have been widely tested preparing various diblock (DB) [15], and triblock (TB) copolymers [15] for drug delivery technologies. Investigators have applied various block copolymers combinations such as PLGA-PEG-PLGA [16], PEG-PLA-PEG [17,18], for the development of sustained release formulations. Several of these polymers are incorporated in microparticle [19], nanoparticle [14], and liposomal preparations [20] for long-term drug release. Recently, the FDA has approved a DEX intravitreal implant for the treatment of macular edema following retinal vein occlusion, diabetic macular edema, or non-infectious uveitis [9,21].

Several attempts have been made to overcome the initial burst of therapeutic molecules using DB or TB copolymers. However, to overcome the above problem, there is an unmet need of designing the optimized block copolymer based delivery system to provide continuous delivery of corticosteroids for longer duration with the minimal burst release. In this regard, pentablock (PB) copolymers were designed to overcome the limitation of the burst release associated with the NP and to provide long-term delivery of therapeutic molecules [22,23]. In this approach, the *in vitro* drug release profile was optimized by adjusting the block length, arrangement, and the ratio of the PCL/PLA/PGA with PEG. The arrangements can be further optimized by changing the molecular weight (MW) of each polymeric block. Considering these facts, a novel PB copolymer (PGA-PCL-PEG-PCL-PGA) was developed to encapsulate DEX in PB-NPs attempting to achieve a long-term delivery. The PB copolymer displays a unique block arrangement, ratio and MW, which can influence the drug release profile of hydrophobic molecules. The purpose of the present study is to examine the DEX release profile of PB copolymer in physiological solution and *in vitro* cell culture media in the presence of human trabecular meshwork (HTM) cell. In addition, the activity and safety over time in ocular cell culture were examined using primary cultures of HTM cells. The approach developed here will be applied to generate an animal model for corticosteroid induced ocular hypertension.

Materials & methods

Materials

Poly(ethylene glycol) (PEG 1 kDa), poly(vinyl alcohol) (PVA), stannous octoate, and dexamethasone (DEX) were obtained from Sigma-Aldrich (MO, USA). The ε-caprolactone, glycolide and L-lactide were procured from Acros Organics (NJ, USA). HPLC solvents and other reagents utilized in this study were of analytical grade.

Methods

Synthesis of copolymers

Novel PB copolymer, poly(glycolic acid)-poly (caprolactone)-poly (ethylene glycol)-poly (caprolactone)-poly (glycolic acid) (PGA-PCL-PEG-PCL-PGA) was synthesized in two steps by sequential ring-opening polymerization reaction [23]. PEG (1 kDa) was utilized as the macroinitiator and stannous octoate act as the catalyst. In the first step, TB copolymer PCL-PEG-PCL was synthesized by polymerization of ε-caprolactone on two open hydroxyl ends of PEG. ε-caprolactone and stannous octoate (0.5% w/w) were added to anhydrous PEG and temperature was raised to 130°C. After 24 h, the reaction mixture was dissolved in methylene chloride followed by precipitation in cold ether. Purified TB copolymer was then used for the preparation of PB copolymer. Stannous octoate (0.5% w/w) was added as a catalyst in the reaction mixture containing a predetermined quantity of TB copolymer. The synthesis of PB copolymer was carried out at 130°C for 24 h under inert atmosphere. After 24 h, the reaction mixture was dissolved in methylene chloride followed by precipitation in cold petroleum ether. The purified PB copolymer was vacuum-dried and stored at -20°C until further analysis. Reaction schemes for the synthesis of TB and PB copolymers were depicted in Figure 1A & B, respectively.

Characterization of copolymers

The synthesized TB and PB copolymers were characterized for their MW, polydispersity index (PDI), and purity by proton (^{1}H) nuclear magnetic resonance (^{1}H-NMR) spectroscopy, gel permeation chromatography (GPC) and powder x-ray diffraction (PXRD). The structures and MWs of copolymers (TB and PB) are described in Table 1.

^{1}H-NMR spectroscopy

^{1}H-NMR spectra of TB and PB copolymers were acquired on a 400 MHz NMR instrument (Varian Inc., CA, USA). The chemical shift (δ) values were reported in parts per million (ppm). NMR samples were prepared by dissolving TB and PB copolymers in deuterated chloroform in a 5 mm outer diameter NMR tubes (Wilmad-LabGlass, NJ, USA).

GPC analysis

The purity, MW and PDI of TB and PB copolymers were further analyzed by GPC analysis. Polymer samples were analyzed with Waters 410 refractive index (RI) detector (Waters, MA, USA). Briefly, samples

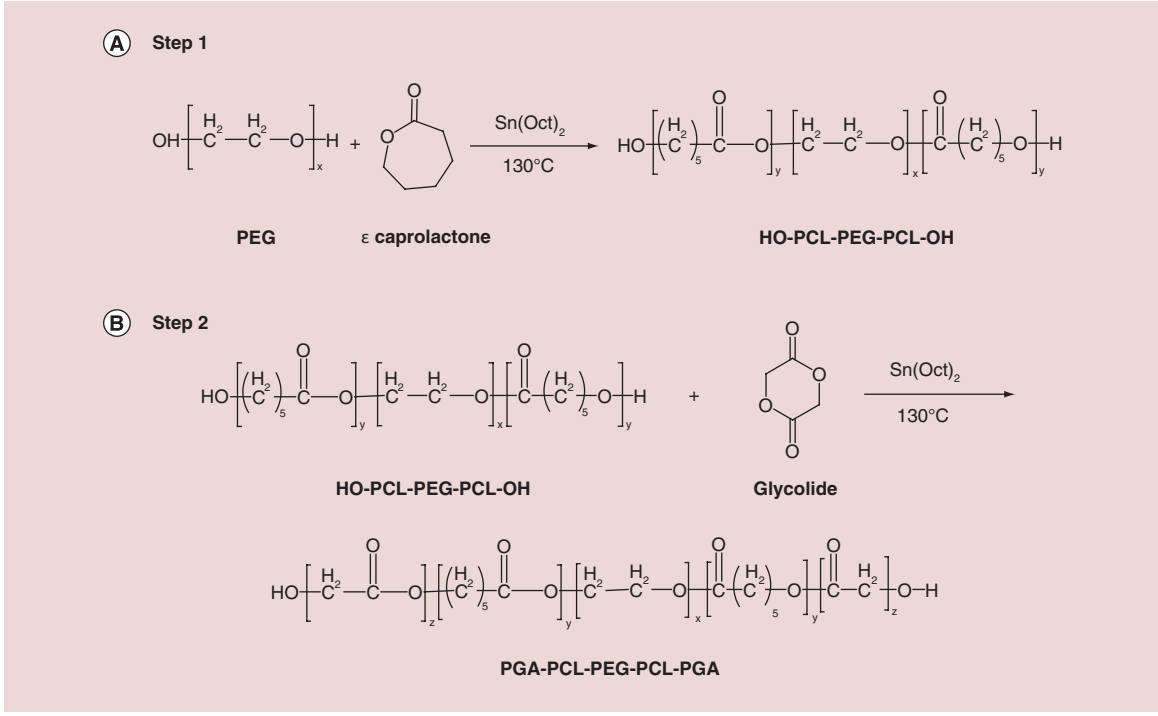


Figure 1. Synthesis scheme for (A) triblock (TB: PCL-PEG-PCL) copolymer and (B) pentablock (PB: PGA-PCL-PEG-PGA-PCL) copolymer by ring opening bulk copolymerization method.

were prepared by dissolving 1 mg of copolymers in tetrahydrofuran (THF) (THF was utilized as eluting agent at the flow rate of 1 ml/min). Separation was carried out on a Styragel HR-3 column (Waters, MA, USA). The internal and external temperatures of the SEC column were maintained at 35°C using a Waters column heater module controlled by 410 RI detector. The data were acquired and processed with Waters Millenium[32] software (version 3.2). A calibration curve was prepared by using Dextran SEC standards (Polymer Standards Service-USA, MA, USA) in the MW range of 5.2–410 kDa. A volume of 200 µl was injected into the SEC system in each analysis.

PXRD analysis

To analyze the crystallinity of copolymers, PXRD analysis was performed using Rigaku MiniFlex automated x-ray diffractometer (Rigaku, TX, USA) equipped with Ni-filtered Cu-Kα radiation (30 kV and 15 mA). The analysis was performed at room temperature at the scanning rate of 5°/min.

Formulation of DEX-encapsulated PB nanoparticles (DEX-PB-NPs)

DEX-loaded PB copolymer NPs were prepared by oil-in-water (O/W) single emulsion solvent evaporation method. Briefly, DEX (5 mg) and PB copolymer (25 mg) were dissolved in methylene chloride copolymers to make the organic phase. The aqueous phase was comprised of 5 ml of 2% PVA. The O/W emulsion was formed using probe sonication. The organic phase was then evaporated by stirring the emulsion overnight. NPs were separated by ultra-centrifugation at 20,000 rpm for 45 min at 4°C. NPs were washed twice with distilled deionized water (DDW), and centrifuged to remove the traces of PVA and unentrapped

Table 1. Characterization of block copolymers.

Copolymers	Structure	Total Mn† (theoretical)	Total Mn‡ (calculated)	Total Mn§ (calculated)	Mw§ (GPC)	PDI§
TB	PCL_{7000}-PEG_{1000}-PCL_{7000}	15,000	14,278	12,289	17,562	1.83
PB	PGA_{3000}-PCL_{7000}-PEG_{1000}-PCL_{7000}-PGA_{3000}	21,000	20,264	17,952	23,158	1.36

†Theoretical value, calculated according to the feed ratio.
‡Calculated from ^{1}H-NMR.
§Determined by GPC analysis.
GPC: Gel permeation chromatography; NMR: Nuclear magnetic resonance; PDI: Polydispersity index.

DEX. The purified NPs were freeze-dried with mannitol (50 mg) as a cryoprotectant and stored at -20°C until further use.

Physicochemical characterization of NPs

Size distribution measurements

DEX-PB-NPs were analyzed for their particle mean diameter: nm, and size distribution by nanoparticle tracking analysis (NTA) using a Nanosight LM10 instrument (Nanosight, Salisbury, UK). Freeze dried DEX-PB-NPs (1 mg/ml) suspended in DDW were subjected to particle size analysis at room temperature and 90° scattering angle. All the samples were analyzed in triplicate (n = 3).

Entrapment efficiency & drug loading

The entrapment efficiency (EE) (%) and drug loading (DL) (%) were estimated by the ultrafast liquid chromatography (UFLC) assay for the amount of DEX in the supernatants obtained from the NP preparation. Equations 1 and 2 were used for the calculation of EE (%) and DL (%), respectively.

$$EE(\%) = \left(1 - \frac{\text{Amount of drug in supernatant}}{\text{Total amount of drug}}\right) \times 100$$

$$DL(\%) = \left(\frac{\text{Amount of drug in nanoparticles}}{\text{Total amount of drug and polymer}}\right) \times 100$$

In vitro drug release profile of DEX-PB-NPs

To analyze the *in vitro* drug release profile, 1 mg of DEX equivalent freeze-dried NPs were suspended in a dialysis tube. DEX-loaded NPs were suspended in 25 ml of phosphate-buffered saline (PBS) pH -7.4 at 37°C. The tube containing dialysis bag was placed in a water bath at 37°C (GFL 3032 Shaker, LABOTECT, Rosdorf, Germany). At predetermined time intervals, 1 ml of clear supernatant was collected and replaced with the same volume of fresh PBS (preincubated at 37°C). Drug concentrations were measured by UFLC analysis. All experiments were conducted in triplicate (n = 3). *In vitro* release data were expressed as cumulative drug released (%) with time.

UFLC assay

Reversed phase UFLC assay was employed to analyze EE, DL and *in vitro* release of the DEX-PB-NPs. A Shimadzu UFLC system (Shimadzu Scientific Instruments, MD, USA) coupled with pumps (LC-20AT), degasser (DGU-20A3R), DAD detector (SPD-20AV) and autosampler (SIL-20AHT) was employed. A Phenomenex column (Phenomenex C18 kinetex column (100 × 4.6 mm, 5 mm) was used at a total flow rate of 0.5 ml/min. An isocratic elution method was employed for the separation with mobile Phase A (water with 0.1% formic acid) at 60% and mobile phase B (acetonitrile [ACN] with 0.1% formic acid) at 40% were running for 8 min. Concentration of DEX standards ranged from 250 to 0.488 µg/ml prepared in the mobile phase. Injection volume of 30 µl was used for each analysis and the DAD detector was set at 254 nm for DEX quantification.

In vitro tolerability studies of PB copolymer & PB-NPs

To analyze the cytotoxic effects of PB copolymers on corneal, conjunctival and retinal cell lines, cell cytotoxicity (lactate dehydrogenase [LDH]) and cell viability (MTT) assays were performed according to the supplier's instructions (Promega Corp., WI, USA). SV-40 (human corneal epithelial transfected with a recombinant SV-40-adenovirus vector cell), CCL20.2 (human conjunctival epithelial cell/Chang's conjunctival cell line) and D407 (human retinal pigment epithelium cell) cells are immortalized and can be subcultured many times, while maintaining their physiological properties. SV-40 cells were cultured in DMEM/F-12 medium supplemented with 15% (v/v) heat-inactivated fetal bovine serum (FBS), 22 mM $NaHCO_3$, 15 mM HEPES and 5 mg/l insulin, 10 µg/l human epidermal growth factor, 100 mg penicillin and 100 mg streptomycin each. Both cell lines were incubated at 37°C, 5% CO_2 and 98% humidity. CCL20.2 cells were maintained in a cell culture flask containing Minimum Essential Medium (MEM), Earle's Balanced Salt Solution medium supplemented with 10% FBS, 100 U/L of penicillin, 100 mg/l of streptomycin, $NaHCO_3$ (2.2 mg/ml) and 2 mM l-glutamine. D407 cells were grown at 37°C, humidified 5% CO_2/95% air atmosphere in a DMEM culture medium supplemented with 10% (v/v) FBS (heat inactivated), 29 mM $NaHCO_3$, 20 mM HEPES, 100 mg of penicillin and streptomycin each, and 1% nonessential amino acids at pH 7.4. The cells were harvested at 80–90% confluency with TrypLE™ Express (a superior replacement for trypsin) [15,24].

Human trabecular meshwork (HTM) cell culture

Five strains of trabecular meshwork cells (HTM120, 136, 126, 134 and 141) were isolated from eyes of human donors of ages 11 and 3 months old (HTM120 and 136), 88, 51 and 38 years old (HTM126, 134 and 141), respectively, with no documented history of eye disease. The genders for the HTM cells

were HTM126, 136: females; HTM134, 141: males; HTM120: unknown, no records available from the Eye Bank. Cells were isolated and characterized as previously described [25,26]. Human eye tissues were sourced ethically from Miracles in Sight (NC, USA), accredited by the Eye Bank Association of America. The research uses of eye tissues were in accordance with the terms of the informed consents of the donors and/or donor family. HTM cells (passages 3–6) were seeded into 24- or 96-well culture plates in DMEM containing 10% FBS (Atlanta Biologicals, GA, USA) until cells reached confluency. As a differentiation step, the cells were then switched to DMEM medium containing 1% FBS for at least 7 days prior to experimentation.

Dexamethasone nanoparticle (DEX-PB-NPs) treatment

HTM cells were treated with PB-NPs (1 mg/ml) containing a total of 23 µg DEX (DEX-PB-NPs) or Con-NPs (without DEX), DEX (39.25 ng/ml) or control (Con: 0.1% ethanol) in fresh 1 ml DMEM containing 1% FBS (1% DMEM). Two days after initial treatment, cell culture supernatant was removed and replaced with fresh 1% DMEM medium containing either 0.1% ethanol vehicle or DEX (39.25 ng/ml in 0.1% ethanol). Similarly, medium in Con-NPs or DEX-PB-NPs treated wells were replaced with fresh 1% DMEM. Then, the cell culture supernatant was collected and replaced with fresh 1% DMEM once/week for a total of 12 weeks for DEX-NP or Con-NP treatment wells. Other wells were replaced with fresh 1% DMEM medium containing either Con or DEX once/week. After 4 weeks, all wells were replaced with fresh 1% FBS medium once in a week for an additional 8 weeks and stored at -80°C until further analysis.

Western blot analysis

Secreted MYOC levels in cell culture supernatants were detected and normalized following our previously published methods [27]. Briefly, cell culture supernatants were collected from wells of culture plates after 2 days of treatment, and then once/week for 12 weeks. At the end of 12 weeks, cells were harvested and rinsed twice with cold PBS. Cells were scraped into 80 µl of lysis buffer (25% glycerol, 0.0625M Tris.HCl, 2% SDS) containing 5% beta mercaptoethanol. Cell culture media at each time point was mixed with 4 × loading buffer (50% glycerol, 0.125M Tris.HCl, 4% SDS) containing 5% beta mercaptoethanol and boiled for 10 min before storing at -20°C. For WB analysis, 24 µl of solubilized proteins in the cell culture supernatant containing 4 × loading buffer were loaded into 8% polyacrylamide gel slabs; and 10 µl of cell lysates were loaded to 10% polyacrylamide gel slabs. The proteins were separated via SDS-PAGE. Fractionated proteins were then transferred electrophoretically to nitrocellulose membranes. Nonspecific binding of antibodies to membranes containing transferred proteins was reduced by incubating with Tris-buffered saline with 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk (blocking buffer).

Rabbit polyclonal antibodies against MYOC or a mouse monoclonal antibody against beta-actin (Sigma-Aldrich, MO, USA) in blocking buffer were incubated overnight with membranes at 4°C. The next day, the membranes were first washed in TBS-T (three times for 10 min) and then were incubated in blocking buffer containing horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., PA, USA) for 1 h. After incubation, membranes were washed with TBS-T as before. Protein-antibody complexes were visualized using a chemiluminescent horseradish peroxidase (HRP) antibody detection reagent spray (HyGLO; Denville Scientific, Inc., NJ, USA) and exposure to x-ray film (Phonix Research Company, NC, USA). The protein abundance in each band was quantified by densitometry using ImageJ image analysis software (GeneSnap/GeneTools, Syngene, MD, USA).

Cell cytotoxicity assay

The LDH assay was performed using previously published protocol with minor modifications [22]. Briefly, 5, 25 and 50 mg/ml of PB copolymer were dissolved in ACN and 100 µl was aliquoted in each well of the 96-well plate. Plates were exposed overnight under UV light (laminar flow) for polymer sterilization as well as evaporation of ACN. Similarly, 5, 25 and 50 mg/ml of PB-NP was dispersed in culture media and filtered through 0.22 µM filter. One hundred microliter of this solution was added to the 96-well cell culture plate. D407, SV-40 and CCL.20.2 cells at the density of 1.0×10^4 were seeded in each well and incubated at 37°C, 5% CO_2 in a humidified atmosphere for 48 h. After completion of the incubation period, cell supernatants were analyzed for the quantification of LDH release using the LDH assay kit (Takara Bio, Inc., Otsu, Japan). Absorbance of each well was measured at 450 nm using a DTX 800 Multimode microplate reader (Beckman Coulter, CA, USA). The LDH release (%) was calculated according to Equation 3 and more than 10% of LDH release was considered as cytotoxic.

$$\text{LDH release (\%)} = \frac{\text{Abs. of Sample} - \text{Abs. of negative control}}{\text{Abs. of positive control} - \text{Abs. of negative control}}$$

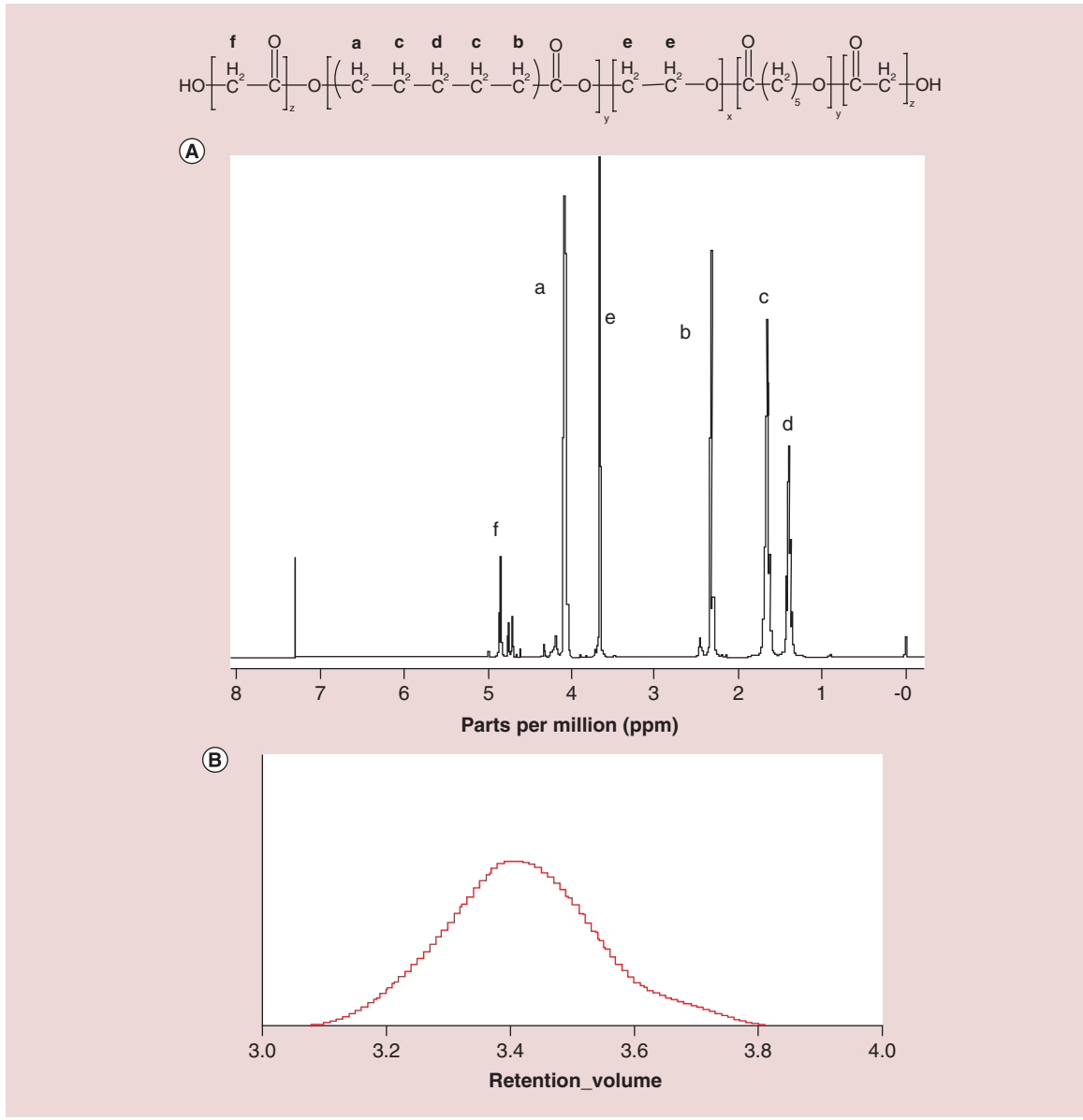
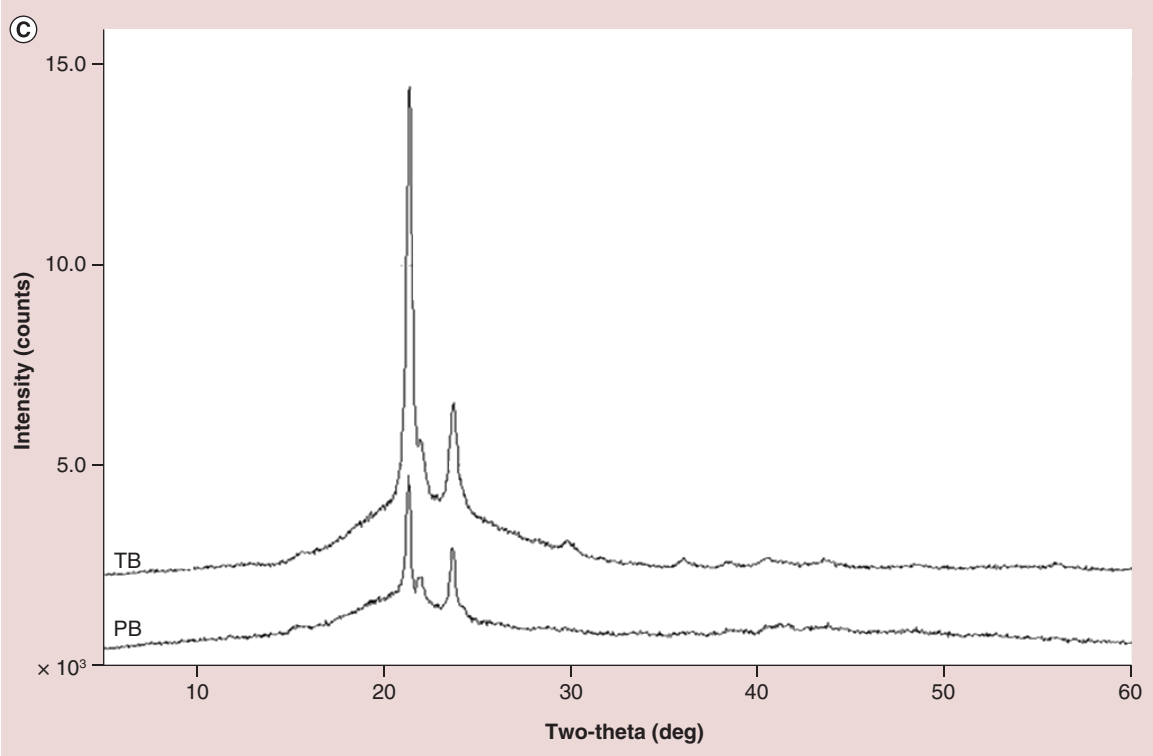


Figure 2. Charcaterizations of pentablock copolymers (cont. on facing page). (A) Proton nuclear magnetic resonance (^{1}H NMR) spectra depicts peaks comprised of glycolic acid which displayed a series of singlets between 4.6 and 4.9 ppm confirming methylene protons of PGA block; **(B)** GPC depicts a single peak indicating mono distribution of molecular weight; **(C)** PXRD patterns of TB and PB copolymers exhibit crystalline peaks of PCL at 2θ = 21.5° and 23.9°.
GPC: Gel permeation chromatography; PB: Pentablock; PCL: Polycaprolactone; PXRD: Power x-ray diffraction; TB: Triblock.

To test the cytotoxicity in HTM cells, three strains of confluent HTM cells (HTM141, HTM126 and HTM136) kept in 1% DMEM media for 1 week were treated with Con vehicle (0.1% ethanol), DEX (39.25 ng/ml in 0.1% ethanol), Con-NPs (1 mg/ml) or DEX-PB-NPs (1 mg/ml) in 1% DMEM media. The treatment protocol was the same as for collecting media for MYOC analysis. At the end of 12 weeks of incubation, cytotoxicity was determined by measuring LDH release from the cells using an LDH assay kit (Roche, IN, USA). Briefly, the supernatant was carefully removed, centrifuged and transferred to a 96-well plate. The cells were lysed and then transferred to a 96-well plate. A reaction mixture consisting of catalyst/dye combination was prepared, and 100 µl was added directly to each of 100 µl of the cell supernatant, cell lysates, plain media and lysate solution. After incubation at 25°C for 30 min, absorbance was mea-



sured using a spectrophotometer at 490 nm with a reference wavelength at 690 nm. Released LDH in the cell supernatant was calculated by subtraction of media background and then normalized by total LDH in cell lysates from each treatment group.

Cell viability assay

The safety and biocompatibility of PB copolymers was further established by performing an *in vitro* cell viability assay (MTS; (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) tetrazolium reduction). MTS assay (Promega Corp., WI, USA) was performed according to a previously reported protocol with minor modifications [22]. PB copolymer solutions at the concentration of 5, 25 and 50 mg/ml in ACN were prepared, aliquoted and sterilized. Following sterilization, D407, SV-40 and CCL20.2 cells were seeded in each well of 96-well plate at a cell density of 1.0×10^4, and incubated at 37°C and 5% CO_2 in a humidified atmosphere for 48 h. Similarly, 5, 25 and 50 mg/ml of PB-NP solution was prepared in culture media and filtered through 0.22 µM filter. From this, 100 µl was added to the 96-well plate. At the end of the incubation period, cell culture medium was aspirated and the cells were incubated for 4 h (37°C and 5% CO_2) in the presence of 100 µl of serum-free medium containing 20 µl of the MTS solution. The absorbance was measured at 450 nm using the above microplate reader. The percent (%) cell viability was calculated using Equation 4. The PB copolymers exhibiting more than 90% of cell viability were considered suitable for ocular applications.

$$\text{Cell viability (\%)} = \frac{\text{Abs. of Sample} - \text{Abs. of negative control}}{\text{Abs. of positive control} - \text{Abs. of negative control}} \times 100$$

To test the cell viability of HTM cells with long-term DEX, Con-NPs or DEX-PB-NPs, a colorimetric assay was performed based on the cleavage of the tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1.3-benzene disulfonate) by mitochondrial dehydrogenases in viable cells (Roche, Mannheim, DE, Germany). Twelve weeks after initial treatment, the WST-1 solution (10 µl/well) was added to each well containing 100 µl of cell culture supernatant. Cells were further incubated for 30 min at 37°C. The plate was read on a spectrophotometer at 440 nm with a reference wavelength of 690 nm.

Extraction method of DEX from cell culture media

Cell culture medium samples were analyzed using a UFLC method as described earlier. Sample preparation was carried out using liquid–liquid extraction technique. Hydrocortisone (HC), a corticosteroid, similar in structure to DEX was used as an internal standard for analysis. Briefly, samples were thawed at room temperature and 50 µl of internal standard was added to the samples (300 µl). Solutions were vortexed for 30 s,

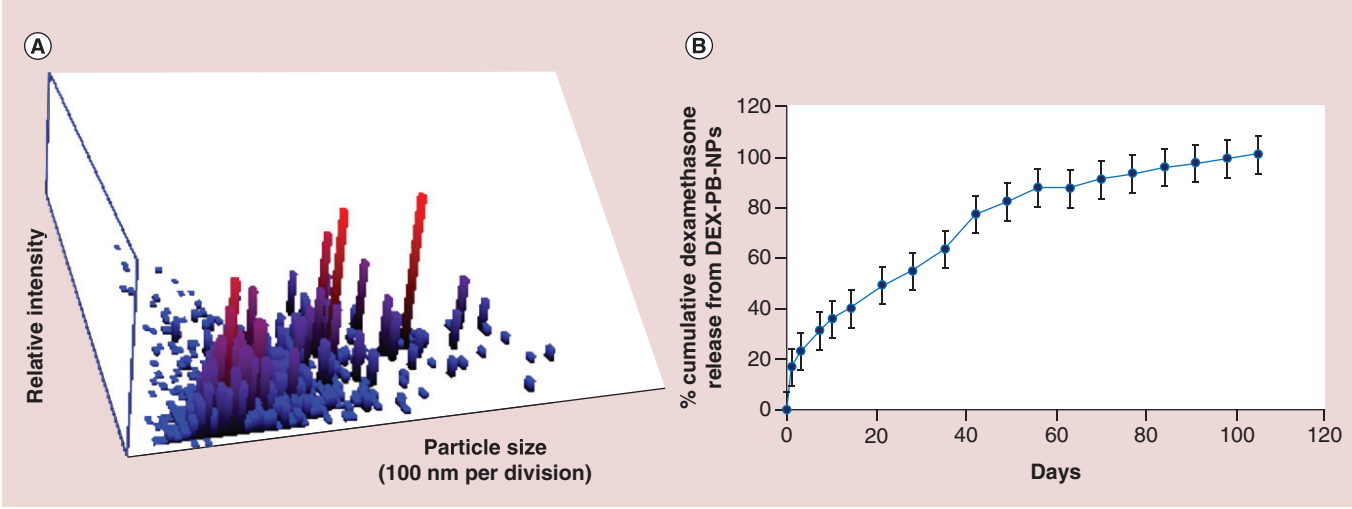


Figure 3. Nanoparticle size distribution and drug release analysis of dexamethasone-encapsulated-pentablock-copolymer-nanoparticle. (A) Particle size distribution of DEX-PB-NPs by NTA. The particle size was in the range of 109 ± 3.77 nm (n = 3). **(B)** *In vitro* release of dexamethasone (DEX) from DEX-PB-NPs. Representative graph shows the long-term release of DEX from DEX-PB-NPs.
DEX-PB-NP: Dexamethasone-encapsulated-pentablock-copolymer nanoparticle; NTA: Nanoparticle tracking analysis.

to which 100 µl of ACN was added, and again vortexed for 30 s to deactivate the serum proteins and enzymes. Each sample was mixed with 300 µl of organic solvent and vortexed again for another 2.5 min to allow equilibration between the phases. For efficient separation of the aqueous and organic layers, samples were extracted twice and centrifuged at 10,000 rpm for 7 min. Aliquots (500 µl) were collected and dried under reduced pressure for 45 min. The residue was reconstituted with 300 µl of mobile phase (ACN [40%] and water [60%]), vortexed for 30 s and transferred into a prelabeled UFLC autosampler vial with silanized inserts. A 30 µl of the resulting solution was injected into the UFLC system and analyzed for DEX quantification.

Statistics

Mann–Whitney U test was used to analyze the statistically significant differences between groups. A p-value of ≤ 0.05 was considered statistically significant.

Results & discussion

Synthesis of PB copolymer & formulation of DEX-PB-NPs

Characterization of PB copolymer

PB copolymer designed for the preparation of NPs was successfully synthesized by ring-opening bulk copolymerization. Figure 2A depicts the typical ^{1}H-NMR peaks comprised of glycolic acid which displayed a series of singlets between 4.6 and 4.9 ppm confirming methylene protons of PGA block. A sharp peak at 3.65 ppm was attributed to methylene protons (-CH_2CH_2O-) of PEG. The ^{1}H-NMR characteristic peaks of PCL unit were observed at 1.40, 1.65, 2.30 and 4.06 ppm represented the methylene (–CH2–) protons of -$(CH_2)_5$-, -OCO-CH_2-, and -CH_2OOC-, respectively. Molecular weights (number average MW: Mn) of PB copolymers were calculated from the integration values of ^{1}H-NMR peaks of individual blocks. Moreover, the absence of any additional peaks in the ^{1}H-NMR spectrum confirmed the purity of PB copolymers. MWs (Mn) calculated from ^{1}H-NMR are reported in Table 1.

Purity, MWs (Mn and weight average MW: Mw) and PDI were further evaluated by GPC are represented in Figure 2B. The PDI of PB copolymers was below 1.5, suggesting a narrow distribution of MWs. Moreover, PB copolymers depicted a single peak in GPC chromatogram indicating mono distribution of MW. Calculated MWs appeared to be very similar to the theoretical MWs obtained from feed ratio. Therefore, theoretical MWs were considered instead of calculating MWs subsequently. PXRD study revealed

Table 2. Characterization of dexamethasone-encapsulated-pentablock-copolymer-nanoparticles.

Polymer	Particle size (nm) (n = 3)	Entrapment efficiency (% w/w) (n = 3)	Loading (% w/w) (n = 3)
PB	109 ± 3.77	63.23 ± 2.31	10.53 ± 0.38

PB: Pentablock.

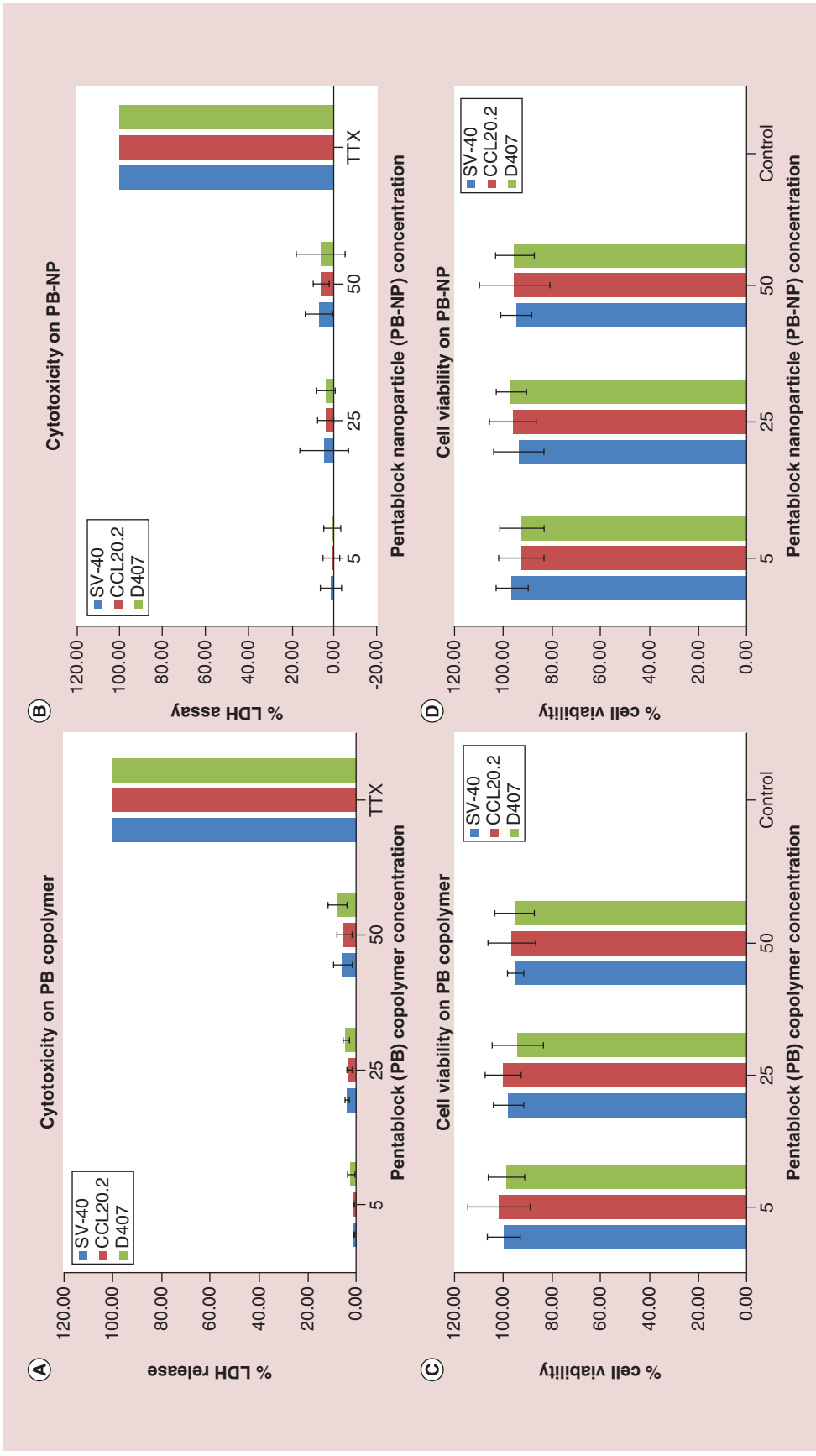


Figure 4. *In vitro* cytotoxicity and cell viability assays. *In vitro* cytotoxicity assay (LDH) of **(A)** pentablock (PB) copolymers; **(B)** blank PB-NPs at different concentrations on D407, SV-40, and CCL20.2 cells. Less than 10% of LDH release was observed after 48 h and 12 weeks exposure, indicating negligible toxicity. *In vitro* cell viability assay (MTS) of: **(C)** PB copolymers; **(D)** blank PB-NPs at different concentrations on D407, SV-40, and CCL20.2 cells. The result demonstrated more than 90% cell viability after 48 hours exposure to PB copolymer.
LDH: Lactate dehydrogenase; MTS: (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2Htetrazolium, inner salt; PB-NP: Pentablock-copolymer-nanoparticle.

Figure 5. Dexamethasone-encapsulated-pentablock-copolymer-nanoparticle induced prolonged MYOC secretion from cultured human trabecular meshwork cells. Representative western blot (WB) images show MYOC secretion over time in response to DEX (39.25 ng/ml), Con-NPs and DEX-PB-NPs treatment. Cells were exposed to NP preparations for entire 12-week period, while only exposed to DEX for first 4 weeks. Rows 1–12 show MYOC protein levels from week 1 to 12. Last row shows beta-actin from the same cells collected at end of 12-week period. n = 5. Four HTM cell strains (HTM120, 126, 134 and 136) showed similar MYOC secretion pattern as representative profile from HTM136 shown.
DEX-PB-NP: Dexamethasone-encapsulated-pentablock-copolymer-nanoparticle; HTM: Human trabecular meshwork; WB: Western blot.

the crystallinity of block copolymers, which are represented in Figure 2C. Interestingly, TB and PB copolymers exhibited crystalline peaks of PCL at 2θ = 21.5° and 23.9°. PXRD patterns of TB and PB indicated that PCL blocks have retained semicrystalline structure, even after covalent conjugation with PGA blocks. Conjugation of PGA blocks at the terminals of TB copolymers slightly affected the intensity of crystalline peak. Previous published reports suggested that the decrease in crystallinity significantly enhances the degradation of block copolymer [22]. Hence, it is anticipated that PB copolymer can display a slower rate of degradation due to its semicrystalline nature.

Characterization of DEX-PB-NPs

Particle size

The particle size of DEX-PB-NPs was approximately 109 ± 3.77 nm (n = 3) observed by NTA reported in Figure 3A.

Entrapment efficiency (EE%) and drug loading (DL%)

DEX-PB-NPs were successfully prepared by single emulsion solvent evaporation method. The percent EE and DL was calculated as 63.23 (± 2.31) and 10.53 (± 0.38), respectively (n = 3) (Table 2).

In vitro DEX release study from DEX-PB-NPs

The release study was performed by suspending 1 mg of DEX equivalent PB-NPs in PBS at 37°C and sampling from the dialysis chamber. Burst release (20%) in 2 days has been observed due to surface bound drug of NPs. Cumulative %DEX released versus time profile is illustrated in Figure 3B. DEX release from the PB-NPs was continuously over 3 months. About 50% of DEX was released within 6 weeks, which appears to be responsible for interactions with PGA chains resulting in a relatively faster release pattern. A biphasic release pattern of DEX release was evident from NPs with an initial burst release, followed by a sustained release phase. DEX demonstrated slow release rate from NP because of the hydrophobicity and low crystallinity of PB copolymer. Hence, PB copolymer-based NPs may be considered more effective relative to existing PLGA and other polymers-based system. An advantage associated with this sustained release formulation (DEX loaded NPs) offers higher drug residence at the site of absorption.

In vitro tolerability of PB copolymer & PB-NP on ocular cell lines

In order to investigate the toxicity of PB copolymer and PB-NPs with the biological system, transformed ocular cell lines (SV-40, CCL20.2 and D407 cells) were treated with 5, 25 and 50 mg/ml of PB copolymer and PB-NPs for 48 h. Primary cultures of HTM cells were treated with 1 mg/ml of PB copolymer and DEX-PB-NPs for

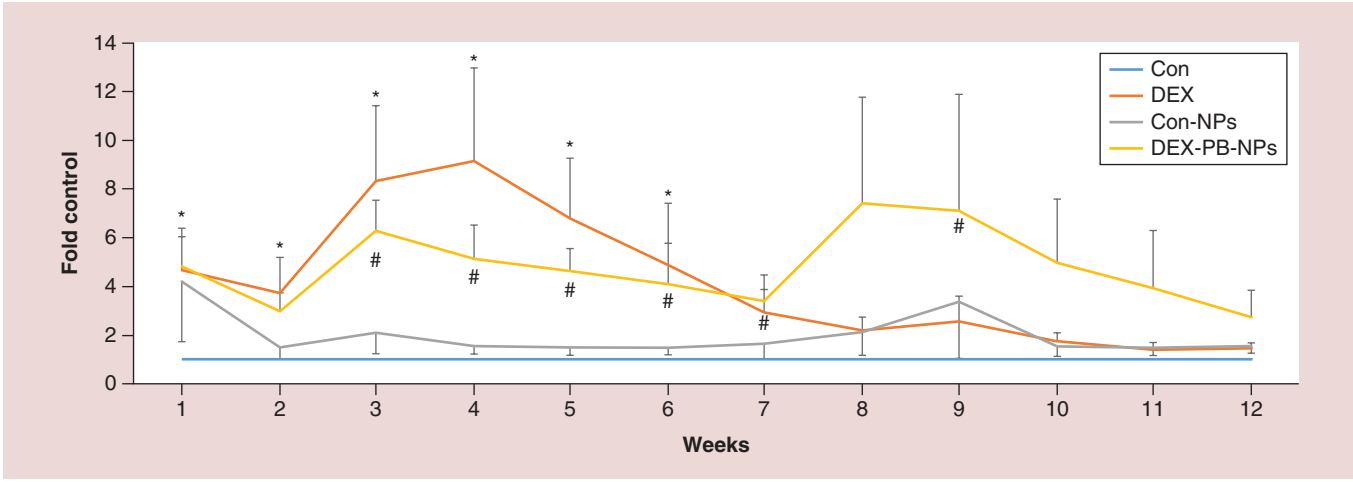


Figure 6. Quantification of MYOC secretion levels in response to dexamethasone-encapsulated-pentablock-copolymer-nanoparticle over time from human trabecular meshwork cells. MYOC western blot images from all five HTM cell strains treated with DEX-NPs, Con NPs or DEX (39.25 ng/ml) for 12 time-points for each strain were digitized and quantified using ImageJ software whereby the band intensities were normalized to β-actin level observed for each individual cell strain. Cells were exposed to NP preparations for entire 12 weeks, while cells were only exposed to DEX for first 4 weeks. The relative MYOC secretion levels from DEX, Con-NPs and DEX-PB-NPs were compared with their individual controls at each time point. The combined data represent mean ± SE, n = 5. Symbols (* and #) indicate the significant differences compared with the Control group and control-NP group, respectively, using Mann–Whitney U Test.
DEX-PB-NP: Dexamethasone-encapsulated-pentablock-copolymer-nanoparticle; DEX: Dexamethasone; HTM: Human trabecular meshwork; NP: Nanoparticle.

12 weeks. LDH is a cytoplasmic enzyme, secreted in cell culture medium following cell-membrane damage. Estimation of LDH concentration in the culture supernatant was used to provide PB copolymer toxicity information. Less than 10% of LDH release was observed after 48 h and 12 weeks exposure, indicating negligible toxicity (Figures 4 & 7A). Noticeably, results were comparable with negative controls.

To further test the cytotoxicity of copolymers, MTS or WST-1 cell viability assay was performed. The WST-1 assay is a rapid and sensitive colorimetric assay based on the cleavage of the tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1.3-benzene disulfonate) by mitochondrial dehydrogenases in viable cells (Roche, Mannheim, Germany). In MTS assay, only metabolically active

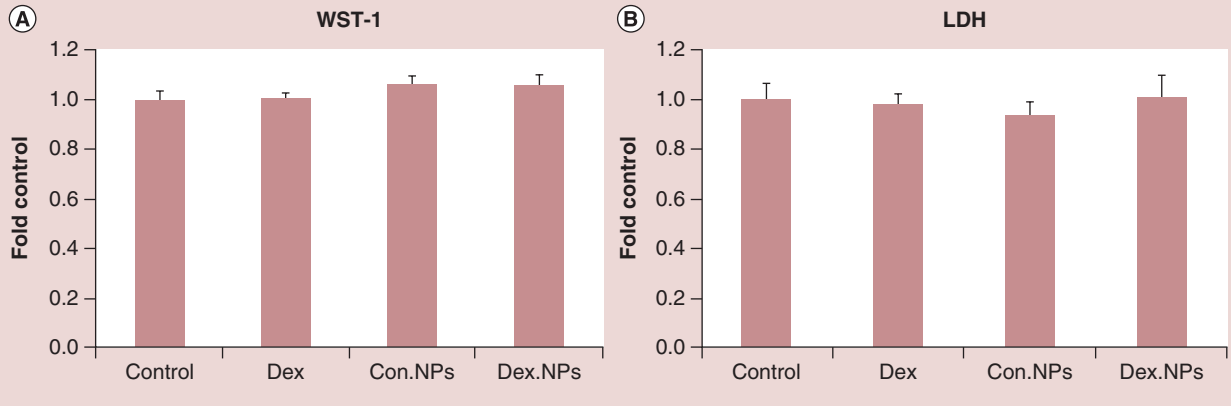


Figure 7. Effect of dexamethasone-encapsulated-pentablock-copolymer-nanoparticle on human trabecular meshwork cytotoxicity over time. Confluent HTM cells were treated with a single application of Con-NPs or DEX-PB-NPs (1 mg/ml). DEX (39.25 ng/ml) treatment was repeated once/week for four weeks. Cell culture media was replaced with fresh 1% FBS media once/week for 12 weeks. At the end of 12 weeks, cell viability was determined by WST-1 and cytotoxicity was examined by LDH release. The relative cell viability and cytotoxicity from DEX, Con-NPs and DEX-PB-NPs treatments were compared with vehicle controls. The data were summarized from three individual cell strains with four replicates in each strain.
DEX-PB-NP: Dexamethasone-encapsulated-pentablock-copolymer-nanoparticle; FBS: Fetal bovine serum; HTM: Human trabecular meshwork.

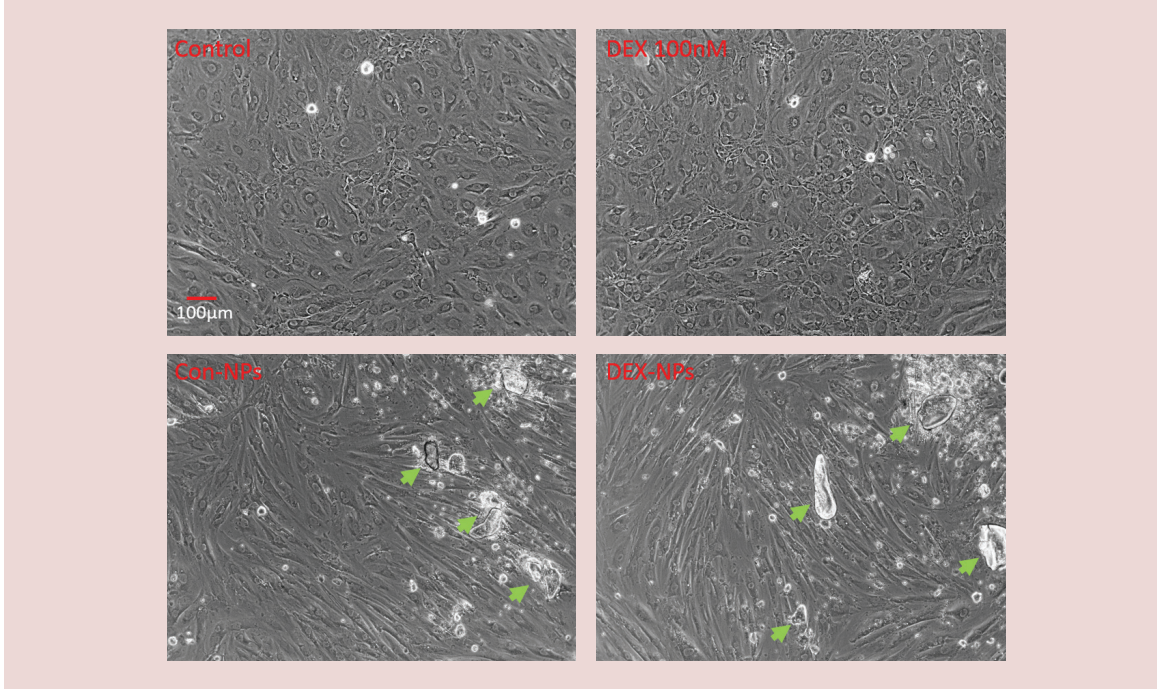


Figure 8. Modification of human trabecular meshwork cell morphology by nanoparticle polymers but not by dexamethasone. Confluent HTM cells were treated with single application of Con-NPs or DEX-PB-NPs (1 mg/ml) for entire 12-week observation period. In contrast, DEX (39.25 ng/ml) treatment was repeated once/week for 4 weeks. Cell culture media was replaced with fresh 1% FBS media once/week for 12 weeks. At end of 12 weeks, cell morphology from each treatment was recorded under phase/contrast light microscope with 10× magnifications. n = 5. Arrows indicate aggregated polymers.
DEX-PB-NPs: Dexamethasone-encapsulated-pentablock-copolymer-nanoparticle; FBS: Fetal bovine serum; HTM: Human trabecular meshwork.

cells convert tetrazolium compound to formazan. Hence, concentrations of formazan products provide a direct estimation of the cell viability. Results in Figure 4 demonstrated that more than 90% cell viability was observed (for all the cell lines) after 48 h and 12 weeks exposure to PB copolymer and PB-NP (Figure 7B). This suggested an excellent safety profile of PB copolymer and nanoformulation for ocular applications.

MYOC secretion from cultured HTM cells

The expression and secretion of MYOC from primary cultures of HTM cells were robustly enhanced following DEX treatment [27–32]. Our previous work [27] suggeste that MYOC was continually upregulated following prolonged 4 weeks DEX treatment and its expression declined over time in the absence of DEX. Thus, the secretion of MYOC can be used as a surrogate read out of biological activity of DEX released from DEX-PB-NPs. A single application of DEX-PB-NPs (1 mg/ml) or control NPs were given to HTM cells. Cell culture supernatant was collected and replaced with fresh 1% DMEM once/week for 12 weeks. DEX or Control vehicle served as controls to compare MYOC secretion levels by WB, being applied once/week for 4 weeks, and then switched to fresh 1% DMEM media for the final 8 weeks. Four HTM cell strains showed similar MYOC secretion patterns, having robust responses for the entire monitoring period (Figure 5). In contrast, one cell strain only responded over a few weeks (Supplementary Figure 1).

Quantitation of WB data from five HTM cell strains (Figure 6) showed that MYOC increased 5.2 ± 1.3, 7.4 ± 4.3 and 2.8 ± 1.1-fold in 4, 8 and 12 weeks in the presence of DEX-PB-NPs compared with 9.2 ± 3.8, 2.2 ± 0.5 and 1.5 ± 0.3-fold in 4, 8 and 12 weeks in the control DEX treatment group (n = 5). There were significant differences at early time points when DEX group is compared with the control group (*). The Dex-PB-NP group was compared with ghost-NP group (#) (Figure 6) using Mann–Whitney U Test. They did not reach statistical significant difference at later time points in DEX-PB-NPs compared with Con-NPs, primarily due to the unresponsiveness of one cell strain HTM141(out of five cell strains) at later time points. These control data are consistent with our previous results [27] where MYOC from cells treated with DEX were significantly upregulated (>4-fold) within the first 6 weeks and then gradually returned to near baseline levels by the end of 6 weeks. Based on the decline in MYOC levels after withdrawal of DEX

from control wells, our data suggested that DEX-PB-NPs releases the biologically active DEX for at least 10 weeks. Interestingly, the first measurement of MYOC levels in Con-NPs-treated groups at week 1 showed a fourfold increase, then dramatically dropped back to near control levels by 2 weeks, where it remained. By comparison, MYOC levels in vehicle treated control wells remained unchanged.

Modification of HTM cell morphology by the PB copolymer

Although not showing any signs of cytotoxicity, the changes in HTM cell morphology were observed in the presence of either NP alone or DEX-PB-NP, becoming more elongated (Figure 8), possibly due to phagocytosis of NPs. A change in morphology was observed as early as 6 weeks and continued up to the last observation at 12 weeks. Interestingly, morphology alterations did not appear to change their MYOC secretion response to DEX released from PB-NPs. In contrast, DEX cells looked similar to vehicle control cells during the 4 weeks of treatment, compared with 12 weeks of Con-NPs. Regardless, changes in morphology were observed in cells treated with Con-NPs alone, suggesting that DEX was not responsible for morphological alterations.

Extraction of DEX from cell culture media

The culture media thawed from different cell strains of HTM cells was collected from cells exposed to DEX-PB-NPs once a week. The DEX was then extracted from the collected media to determine the amount of DEX released from the NPs. The extraction efficiency was found to be more than 90% for both DEX and HC. Interestingly, the amount of DEX released from all three strains found approximately equal and detectable for 12 weeks.

Conclusion

In the current study, novel PB copolymer based DEX encapsulated NPs were synthesized and characterized. It was observed that the location of the PCL block in the middle of the PGA and PEG resulted in high EE and DL. However, positioning of the hydrophilic block (PGA) at the terminal resulted in an initial burst release. Regardless, the burst drug release was less than other block polymers or PLGA formulations studied earlier. In addition, the DEX release from the NPs followed diffusion through the polymer matrices or erosion of the polymeric materials due to the hydrolytic degradation of ester linkages. A possible explanation of enhanced drug release is due to conjugation of PGA to PCL which alters the crystallinity of the PB copolymer. PB copolymer is a semi-crystalline material which provided a longer DEX release owing to its slow degradation. Additionally, the longer DEX release from the PB-NP may be due to high MW of the PB copolymer which required a longer time for degradation.

The activity of a novel PB-NP loaded with DEX was examined by the *in vitro* cell culture system. The MYOC secretion levels in long-term treatment of HTM cells using DEX-PB-NPs were analyzed by WB. With a single application of the DEX-PB-NPs to cultured HTM cells, the robust upregulation of MYOC secretion from HTM cells was detected and maintained at least over 12 weeks. Consistent with our previous study, HTM cells from different donors can vary in response to DEX treatment [27]. Out of five HTM cell strains studied, four of them showed a consistent MYOC increase over the first 6 weeks (4 weeks of treatment). The level of MYOC in the media from these four cell strains decreased afterwards, but was still significantly higher than control levels 7 weeks later. Only one cell strain (HTM141) showed an increase in MYOC secretion in response to DEX-PB-NPs for only 3 weeks before equal to or below control levels for the remainder of the study (Supplementary Figure 1). The varied response to polymer treatments in different cell strains indicated that the polymers should be examined on various strains from multiple donors before the *in vivo* studies.

The individual in response to DEX treatment in the clinic varies; about 40% of non-glaucomatous individuals are steroid responders. The exact mechanism for the steroids-induced IOP elevation is uncertain, but due to its time course likely involves at least two cellular processes in the resistance-generation region of the conventional outflow pathway: increased barrier function of the inner wall of Schlemm's canal and/or alterations in cell contractility and/or extracellular matrix turnover in the TM [33]. Resident TM cells are responsible for maintenance of its unique architecture and their extracellular matrix constituents. The differential response of individual HTM cell strains to DEX and DEX-PB-NPs treatment in our study may partially explain the important role of TM cells in steroids-induced ocular hypertension. For these reasons, it is important that multiple cell strains are tested. Although the PB copolymer did not display any sign of cytotoxicity to HTM cells in this long-term study, it did modify the HTM cell morphology. HTM cell elongation was present in all strains following Con-NPs and DEX-PB-NPs treatment. Morphological modification of HTM cells by the polymers may accompany functional changes that could not be measured in the present study, but should be further investigated. In addition, MYOC elevation was also observed in three HTM cell strains with Con-NPs treatment during the

first week of the treatment. Therefore, the safety of the polymer must be further ascertained.

This study provided evidence that the developed *in vitro* system is a valuable tool for analyzing the safety of polymers as well as biological effects on steroid release from the polymers. Although, the *in vitro* release condition was somewhat different compared with DEX release study with HTM cell strains, an approximately equal amounts of DEX released into the culture media was observed. Under both *in vitro* conditions, DEX was released for a longer period of time. Although, a slow and long-term (>1 month) DEX release was detected in the current study, an early burst release phenomenon still occurred with PB copolymer. It may be acceptable

Summary points

Pentablock copolymer synthesis & characterization

- Novel pentablock (PB) copolymer, poly (glycolic acid)-poly (caprolactone)-poly (ethylene glycol)-poly (caprolactone)-poly (glycolic acid) (PGA-PCL-PEG-PCL-PGA) was synthesized in two steps by sequential ring-opening bulk copolymerization reaction.
- ^{1}H-NMR peaks comprised of glycolic acid displayed a series of singlets between 4.6 and 4.9 ppm confirming methylene protons of PGA block.
- PB copolymers depicted a single peak in gel permeation chromatography chromatogram indicating mono distribution of molecular weight with polydispersity index was below 1.5.
- X-ray diffraction patterns of copolymer indicated that PCL blocks have retained semicrystalline structure, even after covalent conjugation with PGA blocks.

Dexamethasone nanoparticle formulation development, characterization & *in vitro* release

- Dexamethasone (DEX)-loaded PB copolymer based nanoparticles (NPs) were prepared with oil in water single emulsion solvent evaporation method. The percent entrapment efficiency and drug loading were determined as 63.23 ± 2.31 and 10.53 ± 0.38%w/w, respectively. The particle size of NPs was approximately 109 ± 3.77 nm, analyzed by nanoparticle tracking analysis.
- Long-term DEX release from the PB-NPs was found continuous over 3 months. A biphasic release pattern of DEX release was evident from NPs with an initial burst release, followed by a sustained release phase.

***In vitro* cytotoxicity & cell viability of dexamethasone-encapsulated-pentablock-copolymer-nanoparticles**

- The cytotoxic effects of PB copolymers on corneal (SV-40; Human Corneal Epithelial Cell), conjunctival (CCL20.2; Human Conjunctival Epithelial Cell/ Chang's Conjunctival Cell Line), retinal (D407; Human Retinal Pigment Epithelium Cell) and trabecular meshwork (HTM) cell lines, indicating negligible toxicity. In addition, more than 90% cell viability (for all the cell lines) after 48 h and 12-week exposure to PB copolymer and PB-NPs suggested excellent safety profiles.

MYOC elevation: quantitative analysis by western blot

- Five strains of human trabecular meshwork cells (HTM120, 136, 126, 134 and 141) were isolated from eyes of human donors of ages11- and 3-month old (HTM120 and 136), 88-, 51- and 38-year old (HTM126, 134 and 141), respectively, and treated with dexamethasone-encapsulated-pentablock-copolymer-nanoparticles (DEX-PB-NPs). Expression and secretion of MYOC from primary cultures of trabecular meshwork cells are robustly enhanced following DEX treatment and quantitatively analyzed by western blot.
- Based on the decline in MYOC levels after withdrawal of DEX from control wells, our data suggested that DEX-PB-NPs release biologically active DEX for at least 10 weeks. Interestingly, the first measurement of MYOC levels in Con-NPs-treated groups at week 1 showed fourfold increase, then dramatically dropped back to near control levels by 2 weeks, where it remained. By comparison, MYOC levels in vehicle treated control wells remain unchanged.

Conclusion & future prospective

- Activity and safety over time in ocular cell culture were examined using primary cultures of human trabecular meshwork cells.
- The developed system is a valuable and novel tool for determining the safety and effects of steroids released from polymeric NPs.
- Our next goal is to develop the PB copolymers with different molecular weights, ratios and arrangements to provide the controlled zero-rate release without initial burst release.
- PB copolymer did not show any sign of cytotoxicity to HTM cells in this long-term study, it did modify the HTM cell morphology. HTM cell elongation was present in all cell strains after both Con-NPs and DEX-PB-NPs treatment. Morphological modification of HTM cells by the polymers may accompany functional changes that were not measured in the present study, but should be further investigated.
- This approach will be followed to generate an animal model for corticosteroid induced ocular hypertension. The developed system is a valuable and novel tool for determining the safety and effects of steroids released from polymeric NPs.

in some of the *in vivo* studies, but limit its applications especially in clinic. Our next goal is to develop the PB copolymers with different MWs, ratios, and arrangements so that the therapeutic agents could be released in more controlled release fashion without initial burst release. At the same time the PB copolymers must be biocompatible, biodegradable and should not modify the cellular functions and morphology.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/nnm-2017-0140

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