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Novel Thermosensitive Pentablock Copolymers for Sustained Delivery of Proteins in the Treatment of Posterior Segment Diseases

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Abstract

Biodegradable and injectable *in situ* thermosensitive hydrogels were investigated for sustained delivery of protein therapeutics in the treatment of ocular posterior segment neovascular diseases. A series of triblock (TB, polycaprolactone-polyethylene glycol-polycaprolactone (PCL-PEG-PCL), B-A-B) and pentablock copolymers (PBCs) (polylactic acid (PLA)-PCL-PEG-PCL-PLA (C-B-A-B-C) and PEG-PCL-PLA-PCL-PEG (A-B-C-B-A)) were synthesized and evaluated for their thermosensitive behavior. Effects of molecular weight, hydrophobicity and block arrangement on polymer crystallinity, sol-gel transition, micelle size, viscosity and *in vitro* drug release were examined. Results from sol-gel transition studies demonstrated that aqueous solutions of block copolymers can immediately transform to hydrogel upon exposure to physiological temperature. PBC provide significantly longer sustained release (more than 20 days) of IgG relative to TB copolymers. Moreover, kinematic viscosity of aqueous solution at 25°C for A-B-C-B-A type of PBCs was noticeably lower than the TB (B-A-B) copolymers and other PBCs with C-B-A-B-C block arrangements suggesting desired syringeability. The presence of PLA blocks in PBCs (C-B-A-B-C and A-B-C-B-A) significantly reduces crystallinity. Hence, it is anticipated that PBCs will have a faster rate of degradation relative to PCL-PEG-PCL based TB copolymers. PBCs also exhibited excellent cell viability and biocompatibility on ARPE-19 (human retinal pigment epithelial cell line) and RAW-264.7 (mouse macrophage cells), likely rendering it safe for ocular applications. Owing to biodegradability, thermosensitivity, ease of handling and biocompatibility PBC hydrogels can be considered as promising biomaterial for sustained delivery of protein therapeutics to the back of the eye.

Keywords

Block copolymers; controlled drug delivery; *in situ* gelling system; intravitreal; ocular delivery; pentablock copolymers; posterior segment; sol-gel transition; sustained drug delivery; thermosensitive gel

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CONFLICT OF INTEREST I-Novion, Inc. and Genentech, Inc.

INTRODUCTION

Neovascularization is the most common pathology in many blinding disorders including wet age-related macular degeneration (AMD) [1], macular edema (ME) and proliferative diabetic retinopathy (DR) [2]. Retinal microvascular changes have been observed during these complications, which are attributed to retinal vulnerability towards subtle pathological alterations. The role of vascular endothelial growth factor (VEGF) in the development of AMD, ME and DR has been extensively studied and it appears to be a major factor for neovascular growth [3, 4]. Ranibizumab, a Fab fragment of an anti-VEGF antibody has been approved for treatment of ocular neovascularization. Bevacizumab, a chimeric full-length anti-VEGF antibody (149 kDa) approved for treatment of colon cancer is being used off-label for treatment of ocular neovascularization.

Intravitreal injection of bevacizumab has caused reduction in macular thickness, angiogenic leakage and improvement of visual acuity in neovascular AMD patients [5, 6]. However, due to the chronic nature of these diseases and shorter intravitreal half-life of this anti-VEGF antibody, frequent intravitreal injections are indicated to maintain therapeutic activity in retina/choroid. Frequent administrations are inconvenient and cause potential complications such as endophthalmitis, retinal detachment, retinal hemorrhage, and more importantly, patient noncompliance [7–9]. As a number of protein therapeutics are now used in ophthalmology, tumor biology and immunology, pharmaceutical scientists have increasingly focused on the development of novel delivery strategies utilizing current therapeutics rather than the development of new molecules. Sustained release formulations of currently-approved protein therapeutics can be developed more quickly than new molecules while achieving the goal of reduced frequency of intravitreal injections and eventually eliminate the potential complications. This approach may lower the cost of treatment and improve patient compliance.

Recently, various gel forming polymers, sensitive to external environmental stimuli such as pH, temperature, electric field and ionic concentrations have been investigated as sustained delivery systems [10]. In particular, temperature sensitive biodegradable polymers composed of hydrophilic and hydrophobic blocks have drawn more attention. Injectable thermosensitive hydrogel remains in solution phase during injection and, when exposed to body temperature, rapidly phase transforms to a solid hydrogel polymer matrix. The hydrogel matrix protects protein therapeutics from enzymatic degradation and provides sustained release over a longer period of time, eliminating the need for frequent repeated injections. Various biodegradable copolymers composed of hydrophobic polymer blocks including polycaprolactone (PCL), polylactic acid (PLA), polyglycolide (PGA) and poly lactide-co-glycolide (PLGA), and hydrophilic polymer blocks in particular polyethylene glycol (PEG) have been investigated for their thermosensitive behavior [11–13]. Adjustment of the hydrophilic-hydrophobic balance in the block copolymer backbone allows manipulation of the sol-gel transition curve.

Many researchers have investigated thermosensitive TB copolymers (A-B-A or B-A-B) composed of PLA/PCL/PLGA blocks (A) and PEG block (B) for their applicability in the development of sustained delivery formulation [11, 14]. However, PCL based TB

copolymers (A-B-A or B-A-B) as thermosensitive gels appear to be unsuitable due to their poor biodegradability and rapid drug release [15]. The slow degradation of PCL is attributed to its highly crystalline nature [16]. Degradation of PLGA/PLA/PGA produces lactic acid and glycolic acid, which significantly reduces pH in the microenvironment, triggering the degradation of protein therapeutics [17]. Moreover, PLGA also induces structural changes in protein molecules by the process of acylation [17, 18]. Therefore, an alternative polymeric system which reduces the molar mass of lactic acid/glycolic acid upon degradation would be desirable. To date very limited research has been conducted using thermosensitive hydrogels in the treatment of posterior segment neovascular diseases.

In the present study, we have synthesized and evaluated various novel PBC based thermosensitive biodegradable hydrogels. PBCs are composed of biodegradable FDA approved polymer blocks such as PEG, PCL and PLA. This study has addressed four important aspects i.e., synthesis and structural characterization of PCL-PEG-PCL, PLA-PCL-PEG-PCL-PLA, and PEG-PCL-PLA-PCL-PEG block copolymers, effect of molecular weight and block arrangements of polymers on sol-gel transition behavior, *in vitro* cytotoxicity/biocompatibility and *in vitro* release of IgG (a model full length antibody similar to bevacizumab, Mw 150 kDa). Moreover, a possible gelation mechanism of PBCs has been hypothesized and supportive studies are also discussed in this manuscript.

MATERIALS

PEG (1000, 1500, 2000 and 4000), monomethoxy PEG (550), L-lactide, ϵ -caprolactone, stannous octoate, coumarin-6 and lipopolysaccharide were procured from Sigma-Aldrich (St. Louis). Hexamethylenediisocyanate (HMDI) and Micro-BCA™ were obtained from Fisher scientific. Mouse TNF- α , IL-6 and IL-1 β (Ready-Set-Go) ELISA kits were purchased from eBioscience, Inc. Lactate dehydrogenase estimation kit and CellTiter 96® AQueous non-radioactive cell proliferation assay (MTS) kit were obtained from Takara Bio Inc. and Promega Corp., respectively. All other reagents utilized in this study were of analytical grade.

METHODS

Synthesis of TB Copolymers with B-A-B (PCL-PEG-PCL) Block Arrangements

The PCL-PEG-PCL TB copolymers were synthesized by ring-opening bulk copolymerization of ϵ -caprolactone [11]. PEG was utilized as macro-initiator whereas stannous octoate as a catalyst. Briefly, before polymerization, PEG (1, 1.5, 2 and 4 kDa) were vacuum dried for 4 h. Predetermined amount of PEG (4.0 g) and ϵ -caprolactone (8.0 g) were added in the round bottom flask. Polymer melt was degassed under vacuum for 30 min at 130°C. Flask was then purged with nitrogen gas, followed by addition of stannous octoate (0.5 wt%). Reaction was carried out for 24 h at 130°C. The resulting polymer was then dissolved in dichloromethane and precipitated by addition of cold diethyl ether. Precipitates were centrifuged and sediments were vacuum-dried to remove any residual solvents. Purified polymers were stored at -20°C. A schematic synthesis scheme is presented in (Fig. 1a).

Synthesis of PBCs with C-B-A-B-C (PLA-PCL-PEG-PCL-PLA) Block Arrangements

TB copolymer (PCL₁₂₅₀-PEG₁₅₀₀-PCL₁₂₅₀, TB-5) was synthesized as per method described in earlier section for preparation of PBCs 1 (PB-1), 2 (PB-2) and 3 (PB-3) (Table 1). To synthesize PBCs, predetermined amount of TB-5 and L-lactide were added in a round bottom flask and degassed under vacuum for 30 min at 130°C. Flask was then purged with nitrogen gas and followed by addition of stannous octoate (0.5 wt%). Reaction was carried out at 130°C for 24 h. The resulting polymers were purified and stored in a similar manner as described earlier.

Synthesis of PBCs with A-B-C-B-A (PEG-PCL-PLA-PCL-PEG) Block Arrangements

PBCs, PB-4 and PB-5 were synthesized by ring opening copolymerization where mPEG (550) was utilized as macro-initiator and stannous octoate (0.5 wt%) as a catalyst [11]. Firstly, TB copolymers, mPEG-PCL-PLA were synthesized by ring opening copolymerization in a manner described earlier. The resulting TB copolymers were coupled utilizing hexamethylenediisocyanate (HMDI) as a linker to prepare PEG-PCL-PLA-PCL-PEG PBCs. Coupling reaction was carried out at 70°C for 8 h. Polymers were purified by cold ether precipitation and stored at -20°C. A brief synthetic scheme is depicted in (Fig. 1b).

Fourier-Transform Infrared Spectroscopy (FTIR) Analysis

Fourier transform infrared spectroscopy (FTIR) spectra were recorded with a Perkin Elmer SpectrumOne infrared spectrophotometer at a resolution of 4 cm⁻¹ with scan number of 16. FTIR scan was carried out in a range of 4000–650 cm⁻¹. The resulting IR spectra were analyzed with spectrum-v5.3.1 software.

¹H-NMR Analysis

Purity, molecular structure and molecular weight (Mn) of the block copolymers were analyzed utilizing a Varian 400-MHz NMR spectrometer. NMR spectrograms were recorded by dissolving block copolymers in CDCl₃.

Gel Permeation Chromatography (GPC) Analysis

Molecular weights (Mn and Mw) and polydispersity of polymers were examined by GPC analysis. Briefly, 5 mg of polymer was dissolved in 1.5 mL of tetrahydrofuran (THF). Polymer samples were separated on Styragel HR-3 column maintained at 35°C. THF at the rate of 1 mL/min was utilized as eluting solvent. Samples were analyzed by refractive index detector (Shimadzu).

X-ray Diffraction (XRD) Analysis

Physical states of all the synthesized polymers were determined by XRD analysis. TB and PBCs were analyzed at room temperature by MiniFlex automated X-ray diffractometer (Rigaku, The Woodlands, Texas, USA) equipped with Ni-filtered Cu- α radiation (30 kV and 15 mA). The diffraction angle was ranging from 5° to 45° with 1° per min of increment. Jade 8+ (Material Data, Inc, Livermore, CA) was employed to process the diffraction patterns.

Sol-Gel Transition

The sol (flow)-gel (no flow) transition of block copolymers was examined by following a previously published protocol with minor modifications [19]. Briefly, block co-polymers ranging from 15–30 wt% were dissolved in distilled deionized water followed by 12 h of incubation at 4°C. After equilibration, 1 mL of aqueous polymeric solution was transferred in 4 mL glass vial and placed in water bath. The temperature of the water bath was raised gradually from 10°C to 60°C at an increment of 1°C. Vials were kept for 5 min at each temperature. Gel formation was observed visually by inverting the tubes. A physical state with no fluidity for 1 min was considered as gel phase. The temperature at which the solution transforms to gel phase was considered as critical gelling temperature (CGT) and the temperature where polymer starts to precipitate (phase separation) was described as critical precipitation temperature (CPT).

¹H-NMR for Coumarin-6 Loaded Gel

Five mg of PB-1 copolymer was dissolved in either CDCl₃ or D₂O followed by addition of 0.5 mg of coumarin-6 (hydrophobic dye). Samples containing both polymer and hydrophobic dye were subjected to ¹H-NMR analysis. A similar study was performed utilizing PB-5 copolymer.

Micelle Size Analysis

Aqueous solutions of PB-1 copolymer were subjected to micelle size analysis at room temperature utilizing Zeta sizer (Zetasizer Nano ZS, Malvern Instruments Ltd, Worcestershire, UK). PB-1 copolymer concentrations ranging from 0.1 to 5 wt% were investigated without any further dilution.

Viscosity Measurements

Rheological properties of 15wt% aqueous solution of block copolymers were estimated with an Ubbelohde capillary viscometer at temperatures ranging from 5±1°C to 25±1°C. Temperature of the viscometer was maintained with a temperature controlled water bath. Viscosity values are represented as an average of triplicates (kinematic viscosity, cP±1 standard deviation).

Cytotoxicity

Cell Culture—Human retinal pigment epithelial cell line (ARPE-19) were cultured and maintained according to a protocol provided by ATCC. In brief, ARPE-19 cells were cultured in Dubelcco's modified eagle's medium (DMEM)/F-12 containing 10% heat-inactivated fetal bovine serum (FBS), sodium bicarbonate (29 mM), HEPES (15mM), streptomycin (100 mg/L) and penicillin (100 U/L). Mouse macrophage (RAW-264.7) cells were procured from ATCC. RAW-264.7 cells were cultured and maintained in DMEM supplemented with 10% FBS, streptomycin (100 mg/L) and penicillin (100 U/L). Both cell lines were maintained in humidified atmosphere at 37°C and 5% CO₂.

Lactate Dehydrogenase (LDH) Assay—In order to evaluate the cytotoxicity of polymeric materials, various concentrations of block copolymers were exposed to the

ARPE-19 cells or RAW-264.7 cells at density of 1.0×10^4 . Cells were incubated at 37°C and 5% CO₂ in humidified atmosphere for 48 h. After incubation, levels of LDH in cell supernatant were estimated by LDH detection kit. Samples were analyzed at 450 nm by 96-well plate reader. Amount of released LDH is directly proportional to the cytotoxicity of the polymers. In this study, more than 10% of LDH release was considered as cytotoxic. LDH release (%) was calculated according to the following equation,

$$\text{LDH release (\%)} = (\text{Abs. of Sample} - \text{Abs. of negative control}) / (\text{Abs. of positive control} - \text{Abs. of negative control}) \times 100$$

MTS assay—MTS assay was performed according to a previously published protocol with minor modifications [20]. Briefly, ARPE-19 and RAW-264.7 cells at a density of 1.0×10^4 cells per well were seeded in 96-well plate. Cells were incubated for 24 h at 37°C and 5% CO₂ in humidified atmosphere. After incubation, culture medium was replaced with fresh medium containing various concentrations of block copolymers. Cells were further incubated for 48 h. At the end of incubation period, culture medium was substituted with 100 µL of serum free medium containing 20 µL of MTS solution. Cells were then incubated for 4 h at 37°C and 5% CO₂. After 4 h, absorbance of each well was estimated at 450 nm by 96-well plate reader. Polymer concentrations which exhibited more than 90% of cell viability were considered as non-toxic. Percent cell viability was estimated by following equation.

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

Biocompatibility

RAW-264.7 cells were cultured and maintained according to a protocol described in previous section. In order to evaluate *in vitro* biocompatibility of gelling polymers, a previously published protocol was followed with minor modifications [20]. Briefly, 5.0×10^4 cells were seeded per well of 48-well plates and incubated for 24 h. After incubation, cell culture medium was replaced with fresh medium containing various concentrations of block copolymers. After 24 h of incubation at 37°C and 5% CO₂, supernatant of each well was analyzed by ELISA for quantitative estimation of various cytokines (TNF-α, IL-6 and IL-1β). ELISA was performed according to the manufacturer's protocol. Calibration curves for TNF-α, IL-6 and IL-1β were prepared in the range of 10–750 pg/mL, 5–500 pg/mL and 10–500 pg/mL, respectively.

In vitro Drug Release Studies

For the *in vitro* release experiments, 0.5 wt% of IgG was added to 10 mL vials containing 500 µL of 20 wt% aqueous block copolymer solutions. Solutions were gently mixed (~5 min) at 4°C until IgG was dissolved. Vials were incubated at 37°C for 30 min followed by addition of 5 mL 0.01M phosphate buffer saline (PBS, pH 7.4). Throughout the release period, vials were kept in a water bath maintained at 37°C and 60 rpm. At predetermined time intervals, 1 mL of release sample was collected and replaced with fresh PBS (pre-incubated at 37°C). The amount of released IgG was estimated by Micro BCA™ total protein assay kit. To understand the effect of polymer concentration on IgG release, similar

experiments were performed utilizing 15 wt% and 25 wt% aqueous solutions of PB-1 copolymer.

Release Kinetics

In order to investigate release mechanisms, release data were fitted to various kinetic models, i.e., Korsmeyer-Peppas ($M_t/M_\infty = kt^n$), Higuchi ($Q_t = Kt^{1/2}$), Hixon-Crowell ($C_0^{1/3} - C_t^{1/3} = Kt$), First-order ($\text{Log}C = \text{Log}C_0 - Kt/2.303$), and Zero-order ($C = K_0t$). Based on the R^2 value, best fit model was identified. Diffusion exponent (n) of Korsmeyer-Peppas equation was utilized to understand the mechanism of release.

RESULTS AND DISCUSSION

Synthesis and Characterization of Block Copolymers

FTIR spectrum of PB-4 is reported in (Fig. 2). Absorption band at 1725 cm^{-1} and multiple bands ranging $1000\text{--}1300\text{ cm}^{-1}$ established the presence of ester linkages in PB copolymer. Existence of terminal hydroxyl group was confirmed by C-O stretching band at 1092 cm^{-1} and O-H band (stretch) at 3344 cm^{-1} . C-H stretching bands at 2936 and 2865 cm^{-1} depicted presence of PCL blocks. Absorption band at 1534 cm^{-1} (N-H stretching) exhibited the formation of urethane group in PB-4 copolymer.

$^1\text{H-NMR}$ was employed to characterize PCL-PEG-PCL, PLA-PCL-PEG-PCL-PLA and PEG-PCL-PLA-PCL-PEG copolymers. Figure 3 depicts $^1\text{H-NMR}$ spectra of TB-1, PB-1 and PB-4 block copolymers in deuterated chloroform. As described in (Figs. 3a, 3b and 3c), typical $^1\text{H-NMR}$ characteristic peaks were observed at 1.40, 1.65, 2.30 and 4.06 δ ppm representing methylene protons of $-(\text{CH}_2)_3-$, $-\text{OCOCH}_2-$, and $-\text{CH}_2\text{OOC}-$ of PCL units, respectively. A sharp peak at 3.65 δ ppm was attributed to methylene protons ($-\text{CH}_2\text{CH}_2\text{O}-$) of PEG. Typical signals (Figs. 3b and 3c) at 1.50 ($-\text{CH}_3$) and 5.17 ($-\text{CH}-$) δ ppm were assigned for PLA blocks. Whereas, a peak (Fig. 3c) at 3.38 δ ppm was denoted to terminal methyl of ($-\text{OCH}_3-$) of PEG.

The [EO-[CL]-[LA]] molar ratios of final products were calculated from integrations of PEG signal at 3.65 δ ppm, PCL signal at 2.30 δ ppm and PLA signal at 5.17 δ ppm. In case of PB-4 copolymer, PEG signal at 3.38 δ ppm was applied for the calculation of molar ratio.

Molecular weight (M_w and M_n) and polydispersity of polymers were determined by GPC. Typical GPC curves of TB-1, PB-1 and PB-4 are shown in (Fig. 4). A single peak for each polymer was observed describing unimodal distribution of molecular weight and absence of any other homopolymer block such as PEG, PCL or PLA. Moreover, molecular weights of block copolymers were very close to feed ratio. Polydispersity (PD) was also below 1.47 describing narrow distribution of molecular weights. $^1\text{H-NMR}$ and GPC were applied to calculate molecular weight of block copolymers (Table 1). As summarized in (Table 1), experimental values were consistent with theoretical values derived from feed ratio. Hence for simplicity, theoretical values are mentioned in the following text.

In order to evaluate crystallinity and phase composition, all the block copolymers were analyzed for XRD patterns (Fig. 5). No peaks for PEG or PLA were observed for all block

copolymers. Interestingly, only TB-1, TB-2, PB-1 and PB-2 exhibited crystalline peaks of PCL at $2\theta = 21.5^\circ$ and 23.9° , whereas PB-4 and PB-5 were devoid of any such peaks. XRD patterns of TB-1 and TB-2 indicated that PCL blocks retained semi-crystalline structure even after covalent conjugation with PEG blocks. Conjugation of PLA blocks at the terminals of TB copolymers exhibited significant reduction in the intensity of crystalline peak indicating semi-crystalline structures of PB-1 and PB-2. However, PB-4 and PB-5 were devoid of any crystalline peak suggesting amorphous nature of copolymers with A-B-C-B-A block arrangements. Thus, crystallinity of polymer can be easily controlled by the arrangement of polymer blocks in structural backbone. Moreover, previously published reports suggest that decrease in crystallinity significantly enhances degradation of block polymer [16]. Hence, it is anticipated that PB-4 and PB-5 might have faster rate of degradation relative to TB-1, TB-2, PB-1 and PB-2 copolymers.

Sol-gel Transition

The block copolymers reported in this study, are amphiphilic in nature containing hydrophilic block (PEG) and hydrophobic block(s) (PCL and/or PLA). In case of TB copolymers (TB-1 and TB-2), increased total molecular weight by keeping molecular weight ratio constant i.e., PCL/PEG (2:1) did not alter their thermosensitive behavior. However, further increase in molecular weights (TB-3 and TB-4) with the same hydrophobic-hydrophilic block ratio (2:1) reduced aqueous solubility. Longer PCL chains may significantly enhance intermolecular and intramolecular hydrophobic interactions of polymer, outweighing the ability of PEG to solubilize polymer. PB-1 and PB-2 copolymers are soluble in water and exhibit sol-gel transition behavior. However, increasing the molecular weight of the PLA chain (PB-3) exhibited poor aqueous solubility, which might be due to limitation of PEG to solubilize polymer. For all block copolymers, rise in aqueous polymer concentration from 15 to 30 wt% significantly shifted CGT to lower and CPT to higher values.

Effect of Molecular Weight of Block Copolymer—In order to understand the effect of molecular weight of block copolymers, sol-gel transition curves of TB-1 and TB-2 were compared (Fig. 6). With an increase in total molecular weight of block copolymers from 3000 (TB-1) to 4500 (TB-2) CGT descended whereas the CPT ascended to higher values. The temperature range for gel region or area between CGT and CPT at any given concentration was also significantly enhanced. A similar trend was observed for PB-1 and PB-2 polymers, where molecular weight of PLA was raised in PB-2.

Effect of Hydrophobicity of Block Copolymer—The hydrophobicity of block copolymers can be increased by increasing the molecular weight of the block co-polymer while keeping the molecular weight of PEG constant (PB-1 and PB-2) or by substituting the molecular weight of PLA by PCL (TB-2 and PB-1, PB-4 and PB-5). PB-2 has larger chains of PLA relative to PB-1 copolymer suggesting higher hydrophobicity of PB-2 copolymer. It is important to note that PLA block is less hydrophobic relative to PCL block of similar molecular weight. Therefore, TB-2 and PB-5 are more hydrophobic compared to PB-1 and PB-4, respectively. Sol-gel transition curves for PB-1 and PB-2 (Fig. 7a), PB-4 and PB-5 (Fig. 7b), and TB-2 and PB-1 (Fig. 7c) were compared to understand the effect of hydro-

hobicity on the sol-gel behavior of block copolymers. As described in (Fig. 7a), increased hydrophobicity of PB-2 has significantly reduced the CGT and shifted the value of CPT at higher temperature. Similar behavior was observed when the sol-gel transition curves of PB-4/PB-5, and TB-2/PB-1 were compared. Higher hydrophobicity of polymers may enhance the intramolecular and intermolecular hydrophobic interactions even at lower temperature compared to the hydrophilic copolymers which lead to lower CGT. Additionally, these hydrophobic interactions allow more rigid gel matrix and hence delay polymer precipitation at higher temperature (CPT).

Effect of Block Arrangement—In order to understand the effect of block arrangement on thermogelling behavior, sol-gel transition curves of PB-1 (CB-A-B-C) and PB-5 (A-B-C-B-A) were compared (Fig. 8). Interestingly, CGT and CPT for PB-1 copolymer were significantly lower than PB-5 copolymer at any respective concentration. This behavior may be attributed to the different mechanism of gelation of these block copolymers.

Effects of total molecular weight, arrangement of blocks and hydrophobicity of PBCs on sol-gel transition behavior are in agreement with previously published reports of PLGA-PEG-PLGA [21] and PEG-PLGA-PEG [22] copolymer hydrogels.

¹H-NMR of Coumarin-6 Loaded Polymer Solutions

In order to understand the process of polymer solubilization and behavior of sol-gel transition, ¹H-NMR of PB-1 copolymer was carried out in CDCl₃ (Fig. 9a) and D₂O (Fig. 9b) spiked with coumarin-6. Although normally insoluble in water, aqueous solubility was significantly enhanced in the presence of PB-1 copolymer, which may be due to micellization. A sample containing PB-1 copolymer and coumarin-6 in CDCl₃ exhibited sharp peaks representing various protons of PEG, PCL, PLA and coumarin-6. Interestingly, a sample containing PB-1 copolymer and coumarin-6 in D₂O was devoid of signals of coumarin-6 suggesting that coumarin-6 is inside the micellar core. Broad peaks observed at 3.65 δ ppm and 2.30 δ ppm were attributed to the protons of PEG (-CH₂-CH₂-) and PCL (-OCO-CH₂-), respectively. Proton peaks for PLA and coumarin-6 were absent. Similar behavior was observed with the sample containing PB-5 copolymer and coumarin-6 simultaneously dissolved in CDCl₃ (Fig. 9c) or D₂O (Fig. 9d).

NMR analysis carried out in CDCl₃ exhibited sharp peaks for all the protons indicating free movement of polymer chains and coumarin-6 in organic solvent. However, NMR spectra carried out in D₂O exhibited very few broad peaks representing PEG and PCL blocks only. These results suggest that coumarin-6 was located in the core of micelle along with PCL and PLA blocks. Hence, we were able to see very weak NMR signals for PCL blocks due to the restricted molecular movement, and no signals of PLA and coumarin-6. Strong peak of PEG in D₂O was observed indicating location of PEG as corona of micelles. Results from this study suggest that PBCs are solubilized in water via micellization process where core is composed of PCL-PLA and shell is of PEG.

Micelle Size Analysis

Micelle size and its distribution of PB-1 copolymer were evaluated in water by DLS at room temperature as a function of concentration (Fig. 10). As the aqueous concentration of polymer raised from 0.1 wt% to 5 wt%, two peaks (22 nm and 150 nm) were continuously shifted toward higher particle size. Moreover, broader size distribution with the increase of polymer concentration was observed. Interestingly, at 5 wt% polymer concentration, emergence of a third peak with very large micelle size ($\sim 5 \mu\text{m}$) was also revealed. Increase in particle size and polydispersity as a function of concentration clearly indicate the involvement of micellar aggregation mechanism.

Viscosity Measurement

Table 2 describes the kinematic viscosity of 15 wt% aqueous gelling solutions of different block copolymers at various temperatures ranging from 5°C to 25°C. Kinematic viscosities of polymer solutions accelerated with rise in temperature. Interestingly, at any given temperature, increase in molecular weight of block copolymers exhibited higher viscosity (TB-1 and TB-2 or PB-1 and PB-2). Also, viscosity of TB-2 and PB-5 (hydrophobic polymers) were considerably higher relative to PB-1 and PB-4 (hydrophilic polymers), respectively. It is speculated that hydrophobic interactions exerted by PCL or PCL-PLA blocks with large molecular weight and hydrophobic copolymers are significantly stronger at any given temperature relative to small molecular weight and hydrophilic copolymers, respectively. Subsequently, this phenomenon may have elevated the viscosity of aqueous solutions. In addition, as temperature of the solution rises, these hydrophobic interactions also begin to dominate which eventually improve the viscosity of aqueous polymer solution. Interestingly, polymer structure (arrangement of blocks) also exhibited noticeable effect on viscosity. Hence, kinematic viscosity of PB-1 solution was significantly higher compared to PB-5 aqueous solution.

Hypothesis for Gelation Mechanisms

From our results observed in $^1\text{H-NMR}$, viscosity and micelle size analysis, we have hypothesized two different mechanisms of sol-gel transition for different types of block copolymers such as, (i) hydrophobic segments at the terminals (B-A-B (TB-1 and TB-2) or C-B-A-B-C (PB-1 and PB-2)) and (ii) hydrophilic segments at the terminals (A-B-C-B-A (PB-4 and PB-5)). According to this hypothesis, both types of copolymers can be dissolved via micellization where the core is composed of hydrophobic segments and shell is made up of hydrophilic segment. Therefore, as described in (Fig. 11), interaction of PEG with water molecules was dominated at low temperature (4°C) allowing polymer to solubilize (Fig. 11a). To the contrary, elevation in temperature may cause polymer aggregation and initiate the process of micellization. Therefore, we were able to solubilize hydrophobic dye (coumarin-6) in aqueous solution of PB-1 copolymers. B-A-B or C-B-A-B-C copolymers possess hydrophobic terminals and hence may possibly behave differently in aqueous solution relative to A-B-C-B-A types of copolymers. In case of B-A-B or C-B-A-B-C copolymers, during micellization polymer molecules can serve as inter-micellar bridges, where one hydrophobic end of polymer diffused in the core of one micelle and the other end in different micelle (Fig. 11b). With a rise in temperature (up to CGT), progression of

intermicellar bridges may initiate micellar aggregation and eventually enhance the viscosity of solution. Therefore, we have observed significant enhancement in viscosity of each block copolymer solution as a function of temperature (Table 2). Moreover, the numbers of intermicellar bridges are also proportional to the concentration of block copolymers at any given temperature. Micellar aggregation and larger micelle size were observed with increase in polymer concentration (Fig. 10). At CGT, intermicellar bridges and micellar aggregation may be sufficient enough to form opaque hydrogel (Fig. 11c). Further heating of heterogeneous opaque gel resulted in polymer precipitation. It might be due to strong hydrophobic interactions between PCL or PCL-PLA chains which can significantly overcome weak hydrophilic interactions (hydrogen bonds) between PEG and water molecules. This phenomenon may eventually dehydrate PCL or PCL-PLA core leading to collapse of hydrogel structure (Fig. 11d).

A-B-C-B-A (PB-4 and PB-5) types of copolymers may also be solubilized by micellization. However, in case of A-B-C-B-A types of copolymers, micellar arrangements may be lacking in intermicellar bridges. Therefore, such copolymers demonstrated lower viscosity relative to B-A-B or C-B-A-B-C types of copolymers at any given temperature. At CGT, A-B-C-B-A copolymers may aggregate in compact micellar structure resulting in opaque hydrogel. Further heating of gelling solution (above CPT) may made hydrophobic interactions dominated causing dehydrated micelle core which may eventually result in polymer precipitation.

***In vitro* Cytotoxicity**

To study the compatibility between polymer and biological system (*in vitro* cell culture model), various concentrations of block copolymers were exposed to ARPE-19 and RAW-264.7 cells for 48 h. LDH is the cytosolic enzyme, which is secreted into the cell supernatant following membrane damage. Concentration of released LDH provides a direct estimation of polymer toxicity. Results (Figs. 12a and 12b) indicate less than 10% of LDH release at any given concentration for both the cell types. The results were not significantly different than negative controls i.e., cells without treatment.

Results observed in LDH assay were further confirmed by employing MTS cell viability study. In order to study metabolic response, ARPE-19 and RAW-264.7 cells were incubated with various concentrations of block copolymers. Results indicated in (Figs. 13a and 13b) demonstrate that more than 90% of cells are viable even after 48 h of polymer exposure. No significant difference in cell viability is observed relative to negative control. Results obtained from LDH and MTS assay indicated negligible toxicity suggesting excellent safety profile of block copolymers for back of the eye applications.

***In vitro* Biocompatibility Study**

The absence of inflammatory mediators such as TNF- α , IL-6 and IL-1 β secreted in cell supernatant is a good indicator for biocompatibility of block copolymers. These cytokines were measured in the supernatant of RAW-264.7 cells after 24 h of polymer exposure via sandwich ELISA method according to manufacturer's protocol. Our results (Figs. 14a, 14b, and 14c) indicate that even at 20 mg/mL of polymer concentrations, release of cytokines was

negligible with no significant difference to negative control. Negligible release of TNF- α , IL-6 and IL-1 β support the biocompatibility of PBCs.

***In vitro* Release Study**

IgG was selected as a model protein to evaluate the suitability of block copolymers as controlled release delivery systems for ocular indications. *In vitro* release studies were performed by dissolving IgG in 20 wt% aqueous solution of respective block copolymers. To estimate the concentration of IgG, release samples were analyzed by Micro BCA™. *In vitro* release behavior of IgG from various thermosensitive gels is compared in (Fig. 15). Release of IgG was noticeably affected by chemical composition of block copolymers. TB copolymers (TB-1 and TB-2) exhibited significantly higher burst release (~40–45% of initial dose) relative to PBCs (PB-1, PB-2, PB-4 and PB-5) which showed burst release between ~24–31% of initial dose. Moreover, release of IgG from PBC based thermosensitive gels were sustained for more than 20 days, whereas TB copolymers prolonged the release for only ~12–14 days. Partial replacement of PCL with PLA block significantly reduces the crystallinity (XRD data, (Fig. 5) and hydrophobicity of copolymers. This reduction in hydrophobicity of PBCs may have increased the affinity with IgG resulting in prolonged release of IgG from PBCs relative to TB copolymers.

In addition, the effect of polymer concentration on the rate of IgG release was also studied. As depicted in (Fig. 16), 25 wt% of PB-1 copolymer solution exhibited 74.92% of IgG release within 20 days, whereas 20 wt% and 15 wt% gel showed 85.32% and 96.23% of IgG release during the same time period. It appears that an increase in polymer concentration from 15 wt% to 25 wt% significantly retard drug release rate indicating direct relationship of polymer concentration on drug release. At higher polymer concentration, gel may form compact structure with smaller porosity of gel matrix relative to lower polymer concentration. This structural change may lower diffusion of IgG across gel matrix resulting in lower initial burst release and prolong duration of release.

Release Kinetics

In vitro release data were fitted to zero and first order, Korsmeyer-Peppas, Higuchi and Hixson-Crowell models to delineate the kinetics of IgG release (Table 3). The Korsmeyer-Peppas model had the best fit based on R^2 value. The diffusion exponent, n value ranged from 0.272–0.386 for the tested gelling polymers. The n -values below 0.43 indicate diffusion controlled mechanism of IgG release.

CONCLUSIONS

Compositions of PCL-PEG-PCL (B-A-B), PLA-PCLPEG-PCL-PLA (C-B-A-B-C) and PEG-PCL-PLA-PCL-PEG (A-B-C-B-A) block copolymers were successfully synthesized and evaluated for their utility as injectable *in situ* hydrogel forming depot for controlled ocular protein delivery. PBCs exhibit significantly reduced crystallinity. It is anticipated that biodegradability of these novel copolymers will be significantly improved relative to TB copolymers. PBCs with A-B-C-B-A block arrangements are easy to handle at room temperature. Cell viability and biocompatibility studies confirmed that PBCs are superior

biomaterials for ocular delivery. More importantly, PBCs also exhibit significant sustained release of IgG relative to TB copolymers. These outcomes clearly suggest that PBC based controlled drug delivery system may serve as a promising platform not only for back of the eye complications but also for the treatment of anterior segment diseases.

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Figure 1a.

Synthesis scheme for the TB-1, TB-2, PB-1 and PB-2 copolymers.

Figure 1b.
Synthesis scheme for the PB-4 and PB-5 copolymers.

Figure 2.
FTIR spectrum of PB-4 (PEG-PCL-PLA-PCL-PEG).

Figure 3. ^1H NMR of (a) TB-1 (PCL-PEG-PCL), (b) PB-1 (PLA-PCL-PEG-PCL-PLA) and (c) PB-4 (PEG-PCL-PLA-PCL-PEG).

Figure 4. GPC chromatograms for (a) TB-1 (PCL-PEG-PCL), (b) PB-1 (PLA-PCL-PEG-PCL-PLA) and (c) PB-4 (PEG-PCL-PLA-PCL-PEG).

Figure 5.
XRD patterns of block copolymers.

Figure 6.
Sol-gel transition curves of TB-1 and TB-2.

Figure 7.
Sol-gel transition curves of (a) PB-1 and PB-2, (b) PB-4 and PB-5, and (c) TB-2 and PB-1.

Figure 8.
Sol-gel transition curves of PB-1 and PB-5.

Figure 9.

¹H-NMR of PB-1 and coumarin-6 in (a) CDCl₃ and (b) D₂O, and PB-5 and coumarin-6 in (c) CDCl₃ and (d) D₂O.

Figure 10.
Estimation of PB-1 copolymer micelles at various concentrations i.e., 0.1, 0.5, 2 and 5 wt%.

Figure 11.

Possible micellar gelation schemes for B-A-B, C-B-A-B-C types of copolymers in water during the thermoreversible phase transition, (a) 4°C, solution phase, (b) 25°C, polymer solution is clear but polymer self-assemble to form micelles, (c) 37°C, formation of intermicellar bridges resulting in gel formation, and (d) 50°C, exposure of hydrophobic PCL segment to water phase resulting in precipitation.

Figure 12.

In vitro cytotoxicity assay (LDH) of various block copolymers at different concentrations were performed on (a) ARPE-19 and (b) RAW 264.7 cells

Figure 13.

In vitro cell viability assay (MTS) of various block copolymers at different concentrations were performed on (a) ARPE-19 and (b) RAW 264.7 cells.

Figure 14.

In vitro release of (a) TNF-α, (b) IL-6 and (c) IL-1β from RAW 264.7 cells upon exposure to various concentrations of block copolymers.

Figure 15. Effect of block copolymer composition on *in vitro* release of IgG from thermosensitive gels (20 wt%).

Figure 16. Effect of polymer concentrations (15, 20 and 25 wt%) on *in vitro* release of IgG from PB-1 thermosensitive gels.

List of TB and PB copolymers studied.

Table 1

Code	Structure	Total Mn^a (theoretical)	Total Mn^b (calculated)	Total Mn^c (calculated)	Mw^c (GPC)	PDI ^c	Solubility in water
TB-1	PCL ₁₀₀₀ -PEG ₁₀₀₀ -PCL ₁₀₀₀	3000	3052	3428.7	4714.4	1.37	Soluble
TB-2	PCL ₁₅₀₀ -PEG ₁₅₀₀ -PCL ₁₅₀₀	4500	4578	4896.4	6758.3	1.38	Soluble
PB-1	PLA ₂₅₀ -PCL ₁₂₅₀ -PEG ₅₀₀ -PCL ₁₂₅₀ -PLA ₂₅₀	4500	4524	4748.6	6642.7	1.40	Soluble
PB-2	PLA ₅₀₀ -PCL ₁₂₅₀ -PEG ₁₅₀₀ -PCL ₁₂₅₀ -PLA ₅₀₀	5000	4982	5248.6	6903.4	1.32	Soluble
PB-4	PEG ₅₅₀ -PCL ₅₅₀ -PLA ₁₁₀₀ -PCL ₅₅₀ -PEG ₅₅₀	3300	3218	4271.5	6293.6	1.47	Soluble
PB-5	PEG ₅₅₀ -PCL ₈₂₅ -PLA ₅₀₀ -PCL ₈₂₅ -PEG ₅₅₀	3300	3275	4330.4	6104.1	1.41	Soluble
TB-3	PCL ₂₀₀₀ -PEG ₂₀₀₀ -PCL ₂₀₀₀	6000	5873	-	-	-	Insoluble
TB-4	PCL ₄₀₀₀ -PEG ₄₀₀₀ -PCL ₄₀₀₀	12000	11849	-	-	-	Insoluble
PB-3	PLA ₇₅₀ -PCL ₁₂₅₀ -PEG ₁₅₀₀ -PCL ₁₂₅₀ -PLA ₇₅₀	5500	5328	-	-	-	Insoluble

^aTheoretical value, calculated according to the feed ratio.

^bCalculated from ¹H-NMR.

^cDetermined by GPC analysis.

Table 2

Viscosity of thermosensitive gelling solutions (15 wt%) at various temperatures.

	Viscosity (cp) at various temperature				
Block copolymers	5°C	10°C	15°C	20°C	25°C
TB-1	2.45 ± 0.07	2.75 ± 0.02	3.30 ± 0.07	3.76 ± 0.12	-
TB-2	3.31 ± 0.11	3.95 ± 0.09	4.39 ± 0.06	5.30 ± 0.07	-
PB-1	2.85 ± 0.05	3.02 ± 0.09	3.40 ± 0.04	3.95 ± 0.02	-
PB-2	3.56 ± 0.05	4.02 ± 0.14	4.60 ± 0.02	5.85 ± 0.08	-
PB-4	1.92 ± 0.03	2.16 ± 0.05	2.53 ± 0.08	2.76 ± 0.04	3.16 ± 0.12
PB-5	2.07 ± 0.08	2.22 ± 0.06	2.63 ± 0.05	2.97 ± 0.08	3.38 ± 0.11

Table 3

Coefficient of determination (R^2) for various kinetic models for *in vitro* release of IgG.

Block copolymers	Korsmeyer-Peppas		Higuchi	Hixson-Crowell	First-Order	Zero-Order	Best fit model
	R^2	n	R^2	R^2	R^2	R^2	
TB-1	0.995	0.272	0.959	0.904	0.945	0.822	Korsmeyer-Peppas
TB-2	0.992	0.347	0.984	0.965	0.947	0.864	Korsmeyer-Peppas
PB-1	0.997	0.386	0.985	0.954	0.967	0.891	Korsmeyer-Peppas
PB-2	0.997	0.344	0.991	0.981	0.981	0.934	Korsmeyer-Peppas
PB-4	0.989	0.296	0.970	0.957	0.973	0.871	Korsmeyer-Peppas
PB-5	0.988	0.293	0.979	0.943	0.979	0.859	Korsmeyer-Peppas