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Optimization of novel pentablock copolymer based composite formulation for sustained delivery of peptide/protein in the treatment of ocular diseases

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Abstract

This manuscript is focused on the development of pentablock (PB) copolymer based sustained release formulation for the treatment of posterior segment ocular diseases. We have successfully synthesized biodegradable and biocompatible PB copolymers for the preparation of nanoparticles (NPs) and thermosensitive gel. Achieving high drug loading with hydrophilic biotherapeutics (peptides /proteins) is a challenging task. Moreover, small intravitreal injection volume (100 μ L) requires high loading to develop a long term (6 months) sustained release formulation. We have successfully investigated various formulation parameters to achieve maximum peptide/protein (octreotide, insulin, lysozyme, IgG-Fab, IgG, and catalase) loading in PB NPs. Improvement in drug loading can facilitate delivery of larger doses of therapeutic proteins via limited injection volume. A composite formulation comprised of NPs in gel system exhibited sustained release (without burst effect) of peptides and proteins, may serve as a platform technology for the treatment of posterior segment ocular diseases.

Keywords

Biodegradable; intravitreal; nanoparticles; ocular delivery; protein; sustained delivery

Introduction

Age-related macular degeneration (AMD), diabetic retinopathy (DR) and diabetic macular edema (DME) involves neovascularization and blood leakage at the macular region of retina, retinal pigment epithelium (RPE), Bruch's membrane and choriocapillaries. These are primary vision threatening diseases particularly in elderly patients. Active role of choroidal neovascularization (CNV) is reported in pathophysiology of wet-AMD. CNV causes leakage of blood and accumulation of fluids into subretinal space which lead to scar formation and irreversible vision loss (Kulkarni and Kuppermann, 2005). Currently, anti-vascular

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endothelial growth factor (VEGF) treatment including anti-VEGF antibodies such as bevacizumab and ranibizumab are indicated in the treatment of these disease conditions. Bevacizumab (Avastin®) is a full-length (149 kDa) recombinant humanized murine monoclonal antibody specific to all isoforms of VEGF (Ozturk et al., 2011). Ranibizumab (Lucentis®) is a 48 kDa Fab-fragment of a humanized murine anti-VEGF antibody active against all isomers of VEGF (Sayen et al., 2011). Due to chronic nature of these diseases and shorter half-lives of protein therapeutics, intravitreal injections are administered very frequently (Bakri et al., 2007). Such frequent injections are inconvenient and can cause many other complications such as secondary infections (endophthalmitis), retinal detachment, retinal hemorrhage and patient noncompliance (Peyman et al., 2009, Jager et al., 2004, Sampat and Garg, 2010).

Therefore, formulation scientists have focused on the development of a sustained protein delivery system composed of biodegradable and biocompatible polymers. Many polymeric systems such as poly lactide-co-glycolide (PLGA) (Bilati et al., 2005), PCL-PEG-PCL (Jia et al., 2008), PEG-PCL (Pourcelle et al., 2007), PEG-PLA (Lee et al., 2007), PEG-PLGA (Hirani et al., 2014), PLGA-PEG-PLGA and poloxamers (Radivojsa et al., 2013) have been evaluated for the design of controlled delivery of protein, peptide as well as small molecules. Protein molecules are very fragile and sensitive towards external stimuli. Previously published reports suggested that protein/peptide molecules can easily lose biological activity during formulation development, storage and/or release (Perez et al., 2002, Putney, 1998, Kumar et al., 2006, van de Weert et al., 2000). Moreover, acidic degradation products (lactic acid and/or glycolic acid) of biodegradable polymers reduce pH of the formulation (Fu et al., 2000), and may accelerate a process of acylation (Zablotna et al., 2007, Zhang and Schwendeman, 2012) which eventually compromise stability of protein therapeutics. In addition, one of the important challenges is to develop a formulation which can carry large doses and provide constant release for up to 6 months or more.

An ideal formulation for the intravitreal delivery must possess following characteristics, (a) carry therapeutic dose (high loading) in small volume (100 µL) for 6 months or more, (b) provide constant release (zero-order release) throughout the treatment period without any burst effect, (c) easy to inject, (d) ensure stability of protein/peptide, (e) biodegradable and biocompatible, and (f) biodegradation time of formulation should not exceed 1.5 times of release period. In order to develop such formulation, we have synthesized novel biodegradable and biocompatible pentablock (PB) copolymers composed of FDA approved polymeric blocks such as PEG, PCL, and PLA/PGA. In this manuscript, we have studied effects of various formulation parameters on entrapment efficiency (EE), drug loading (DL) and *in vitro* release profile. In order to incorporate large dose of protein therapeutics in a small volume (100 µL), we have optimized nanoparticle (NP) preparation methods to attain maximum possible DL. To achieve constant (zero-order) release and minimize burst release, we have utilized a novel concept of composite formulation where protein/peptide-loaded PB NPs were suspended in PB thermosensitive gel. Moreover, these optimized methods for NP preparation may be applied to encapsulate proteins/peptides with different molecular weights ranging from 1-237 kDa. Biological activity of released proteins/peptides was also determined utilizing enzyme activity assays.

Materials and Methods

Materials

Poly (ethylene glycol) (PEG, 2 kDa and 4 kDa), methoxy-PEG (550 Da), ϵ -caprolactone, poly (vinyl alcohol) (PVA), stannous octoate, lysozyme from chicken egg white and *Micrococcus luteus* were procured from Sigma-Aldarich (St. Louis, MO; USA). Hexamethylene diisocyanate (HMDI), glycolide and L-lactide were obtained from Acros organics (Morris Plains, NJ; USA). Catalase was purchased from Worthington Biochemical Corp. IgG-Fab and IgG were purchased from Athens Research Technology Inc., and Lee Biosolutions, respectively. Octreotide and insulin were procured from China Peptides Co. Ltd. and MP Biomedicals LLC., respectively. Micro-BCA™ kit and catalase colorimetric assay kit were obtained from Fisher scientific and Arbor assays Inc., respectively. All other reagents utilized in this study were of analytical grade.

Methods

Synthesis of PB copolymers—Novel PB copolymers, poly(lactic acid)-poly(caprolactone)-poly(ethylene glycol)-poly(caprolactone)-poly(lactic acid) (PLA-PCL-PEG-PCL-PLA, PB-A/PB-B), poly(glycolic acid)-poly(caprolactone)-poly(ethylene glycol)-poly(caprolactone)-poly(glycolic acid) (PGA-PCL-PEG-PCL-PGA, PB-C/PB-D), and poly(ethylene glycol)-poly(caprolactone)-poly(lactic acid)-poly(caprolactone)-poly(ethylene glycol) (PEG-PCL-PLA-PCL-PEG, PB-E) were synthesized by ring-opening bulk copolymerization (Gou et al., 2010). Briefly, ϵ -caprolactone was polymerized on two open hydroxyl ends of PEG (2 kDa or 4 kDa) utilizing stannous octoate as catalyst (0.5% w/w). The reaction was carried out for 24 h at 130°C in inert environment. Tri-block (TB) copolymer (PCL-PEG-PCL) was dissolved in dichloromethane (DCM) followed by cold-ether precipitation. Purified TB copolymer was then utilized for the preparation of PB copolymer. Predetermined quantities of TB copolymer and L-lactide (PB-A/PB-B) or glycolide (PB-C/PB-D) were added in round bottom flask. Stannous octoate (0.5% w/w) was added in reaction mixture as a catalyst. The synthesis of PB-A/PB-B was carried out at 130°C for 24 h whereas for PB-C/PB-D at 200°C for 24 h. At the end, the reaction mixture was purified for PB copolymers as described earlier. Purified PB copolymers were vacuum-dried and stored at -20°C until further characterization. PB copolymer with thermosensitive properties (PB-E) was also synthesized, purified and characterized according to previously published protocol with minor modifications (Gou et al., 2010). For the synthesis of PB-E, TB copolymer (mPEG-PCL-PLA) was also synthesized by ring-opening bulk copolymerization. Firstly, ϵ -caprolactone was polymerized at the hydroxyl terminal of mPEG (550 Da) followed by polymerization of L-lactide. Resulting TB copolymers were coupled with HMDI as a linker. The coupling reaction was carried out for 8 h at 70°C. The resulting polymer was purified by cold-ether precipitation followed by drying under vacuum. Reaction schemes for the synthesis of PB copolymers are depicted in Figures 1 and 2. The structures and molecular weights of PB copolymers are described in Table-1.

Characterization of polymers—PB copolymers were characterized for their molecular weight and purity by ¹H-NMR spectroscopy and gel permeation chromatography (GPC).

(i) ¹H-NMR: Polymeric materials (5 mg) were dissolved in 600 μ L of CDCl₃ and were analyzed by Varian-400 NMR instrument. Molecular weight (Mn) and purity of the polymers were evaluated from the ¹H-NMR spectrum.

(ii) GPC: Molecular weights (Mn and Mw), purity and polydispersity (PD) were further evaluated by Ecosec HLC 8320 gel permeation chromatography connected with differential refractometer. Briefly, 5 mg of polymeric material was dissolved in tetrahydrofuran (THF) and separated on Styragel HR-3 column. THF was used as eluting solvent and polystyrene samples with narrow molecular weight distribution were considered as standards.

Preparation of thermosensitive gelling solution—To prepare 20 wt% aqueous thermogelling solution, 200 mg of PB-E copolymer was dissolved in 800 mg of phosphate buffer saline (PBS, pH 7.4) by keeping overnight at 4°C. Sol-gel behavior of the aqueous solution was confirmed by vial inverting method reported earlier (Liu et al., 2008). The polymer solution in PBS was exposed to 37°C for 5 min followed by inversion of vial for 1 min, state of no flow was considered as hydrogel.

Preparation of nanoparticles—Protein/peptide-loaded PB-NPs were prepared by W₁/O/W₂ double emulsion solvent evaporation method (Bilati et al., 2003). Briefly, peptide/protein containing aqueous solution (W₁ phase) was emulsified in organic phase (dichloromethane (DCM) comprising PB copolymers) using probe sonication to form W₁/O primary emulsion. The primary emulsion was further emulsified in aqueous phase containing 2% polyvinyl alcohol (PVA) using probe sonication to prepare W₁/O/W₂ double emulsion. Resulting emulsion was diluted with 2% PVA (W₃) under continuous stirring. DCM of the organic phase was then evaporated under vacuum using rotavap to obtain NPs. NPs were separated by ultracentrifugation at 20,000 rpm for 30 minutes (4°C). Particles were washed twice with distilled deionized water (DDW), and centrifuged at 20,000 rpm for 30 minutes (4°C) to remove traces of PVA and untrapped peptide/protein. Purified NPs were freeze-dried with mannitol (5% w/v) and stored at -20°C until further use. Freeze-dried NPs were evaluated for EE, DL and *in vitro* drug release pattern. Process parameters such as phase volume ratio, drug/polymer ratio, and types of polymer were optimized to achieve maximum DL. The detailed process parameters are reported in Table-2.

Characterization of nanoparticles

(i) Particle size: NPs (1 mg/mL) were suspended in DDW and subjected to particle size analysis. Particle size was evaluated at room temperature and 90° scattering angle utilizing Zetasizer (Zetasizer Nano ZS, Malvern Instruments Ltd, Worcestershire, UK). All the samples were analyzed in triplicate and average particle size was reported.

(ii) Entrapment efficiency (EE) and drug loading (DL): Protein/peptide-encapsulated freeze-dried NPs were evaluated for the estimation of EE and DL. Amount of untrapped protein/peptide in supernatant was determined by micro BCA™ protein assay kit following the manufacturer's protocol. A standard curve of respective proteins/peptides (octreotide, insulin, lysozyme, IgG-Fab, IgG and catalase) were prepared in the range of 3.125-200 μ g/mL. Following equations were utilized for the calculation of EE (%) and DL (%).

EE (%) was calculated with Eq. 1

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

DL (%) was calculated by Eq. 2

$$DL(\%) = \left(\frac{\text{Amount of drug in nanoparticles}}{\text{Total amount of drug and polymer}} \right) * 100 \quad \text{Eq. 2}$$

(iii) In vitro release studies: Protein/peptide-loaded freeze-dried NPs were characterized for injectibility and release kinetics. To evaluate back pressure during injection, precalculated amount of NPs dispersed in thermosensitive gel were manually injected in 1.5 mL eppendorf tubes through 29G insulin syringe preincubated at 4°C. Simultaneously, injectibility of DDW, thermosensitive gel solution and NPs dispersion in DDW was evaluated at 4°C. To perform *in vitro* drug release from composite formulation, precalculated amount of NPs was suspended in 100 µL of PB-E thermosensitive gelling solution (20 wt%) maintained at 4°C. The tubes containing PB NP suspension was then incubated at 37°C for 30 min followed by slow addition of 1 mL of PBS (pH 7.4) preincubated at 37°C. At predefined time intervals, 200 µL of clear supernatant was collected and replaced with fresh PBS (37°C). The released samples were evaluated for protein content by Micro BCA™ total protein assay which was performed according to manufacturer's instructions. The experiments were carried out in triplicates and depicted as cumulative drug release (%) against time. Biological activity of lysozyme and catalase were confirmed by enzymatic assays.

Stability evaluation of lysozyme and catalase

Estimation of enzymatic activity of lysozyme: Enzymatic activity of lysozyme in the released samples was estimated by comparing with freshly prepared lysozyme solutions and/or control samples. Controls were made of lysozyme solution incubated at 37°C in PBS (pH 7.4). These solutions were parallel to the *in vitro* release study from composite formulation. In order to determine enzymatic activity of lysozyme, a stock solution of *Micrococcus luteus* (0.01% w/v) was prepared with phosphate buffer (66 mM, pH 6.15) and diluted to achieve absorbance between 0.2 - 0.6 at 450 nm. One hundred µL of samples, standards or controls were mixed with 2.5 mL of *Micrococcus luteus* suspension. A rate of deceleration of absorbance at 450 nm was determined over a period of 4 min at room temperature. Data was plotted for absorbance against time and slope was utilized for the quantification of lysozyme in enzyme unit (EU). The units of lysozyme (active) per milligram of protein were calculated from the following equations 3 and 4 (Tang and Singh, 2009).

$$\text{Units of lysozyme in mL sample} = \frac{(\Delta A_{450 \text{ nm}} / \text{min Test} - \Delta A_{450 \text{ nm}} / \text{min Blank})(df)}{(0.001)(0.1)}$$

Eq. 3

$$\frac{\text{Units of lysozyme}}{\text{mg of sample}} = \frac{\text{Units of lysozyme/mL of sample}}{\text{mg of lysozyme/mL of sample}} \quad \text{Eq. 4}$$

From definition of lysozyme, one unit of enzyme is able to produce $\text{Abs}_{450 \text{ nm}}$ of 0.001 per minute at pH 6.15 and 25°C utilizing *Micrococcus luteus* suspension. A number of 0.1 represent the volume of release samples, standards or controls and df depicts dilution factor. Biological activity observed for release samples were compared with the respective controls at the same time points.

Estimation of enzymatic activity of catalase: Enzymatic activity of catalase was estimated in release samples. Standard samples with known concentration of catalase were prepared in PBS (pH 7.4) and exposed to 37°C along with *in vitro* release samples. Control, test and standard samples were analyzed for catalase activity with colorimetric assay kit. The assays were performed according to manufacturer's protocol. Briefly, standards with known concentrations were prepared in assay buffer. A 25 μL of standard, control or sample was added to 96-well plate containing 25 μL of hydrogen peroxide solution. The resulting mixture was then incubated for 30 min at room temperature. After incubation, 25 μL of colorimetric detection reagent was added in each well followed by addition of 25 μL Horseradish Peroxidase (HRP) reagent. The plate was incubated for 15 min at room temperature and then analyzed by UV spectrophotometer at 570 nm. A reduction in the absorbance is directly proportional to the catalase activity.

Results and Discussion

Synthesis and characterization of PB copolymers

PB copolymers designed for the preparation of NPs and thermosensitive gel were successfully synthesized by ring-opening bulk copolymerization. Molecular weights (M_n) and purity of the PB copolymers were examined by $^1\text{H-NMR}$ spectroscopy. As depicted in Figures 3, 4 and 5, PCL blocks exhibited typical $^1\text{H-NMR}$ peaks at 1.40, 1.65, 2.30 and 4.06 ppm attributed to methylene protons of $-(\text{CH}_2)_3-$, $-\text{OCO-CH}_2-$, and $-\text{CH}_2\text{OOC}-$, respectively. L-lactide containing PB copolymers (PB-A, B and E) demonstrated two $^1\text{H-NMR}$ peaks at 5.17 and 1.50 ppm representing $-\text{CH}-$ and $-\text{CH}_3-$ groups. Similarly, PB-C and PB-D copolymers comprised of glycolic acid displayed a series of singlets between 4.6 to 4.9 ppm confirming methylene protons of PGA block. $^1\text{H-NMR}$ of PB-E copolymer exhibited an additional peak at 3.38 ppm which represented terminal methyl of $(-\text{OCH}_3-)$ of PEG. Molecular weights of PB copolymers were calculated from the integration values of $^1\text{H-}$

NMR peaks of individual blocks [EO]/[CL]/[LA] or [EO]/[CL]/[GA]. Moreover, absence of any additional peaks in ^1H -NMR spectrum confirmed the purity of PB copolymers. Molecular weights calculated from ^1H -NMR are reported in Table 1.

Purity, molecular weight (M_n and M_w) and polydispersity were further evaluated by GPC. Polydispersity of all the polymers were below 1.45 (Table 1) suggesting narrow distribution of molecular weights. These parameters obtained for GPC analysis were very close to the feed ratio. Moreover, block copolymers depicted a single peak in GPC chromatogram (data not shown) indicating monodistribution of molecular weight and absence of any homopolymers such as PLA, PGA, PCL and PEG. The M_n values obtained from GPC analysis were noticeably lower than M_n values observed from ^1H -NMR spectroscopy. This result can be attributed to the difference in hydrodynamic diameter of block copolymers relative to parent homopolymers (Huang et al., 2003). Calculated molecular weights were very similar to the theoretical molecular weights obtained from feed ratio. Therefore, theoretical molecular weights are considered instead of calculated molecular weights subsequently.

Preparation and characterization of nanoparticles

PB NPs were successfully prepared with double emulsion solvent evaporation method ($W_1/O/W_2$). We have attempted to optimize NP preparation method to achieve maximum possible DL. In this section we have investigated the effect of polymer hydrophobicity, salt (NaCl), drug to polymer ratio, concentration and phase volumes, and types of protein/peptide on EE and DL.

Entrapment efficiency (EE) and Drug loading (DL)—IgG-Fab-loaded NPs were prepared utilizing two PB copolymers (PB-A and PB-B) to determine the effect of polymer structure or hydrophobicity on DL where PB-A copolymer is more hydrophobic than PB-B copolymer. As represented in Table 2, EE and DL for PB-A copolymers (Batch 1) were ~54% and 5.62%, respectively whereas PB-B copolymer (Batch 2) exhibited ~46% of EE with 4.99% of DL. NPs composed of PB-A copolymer demonstrated higher DL relative to PB-B NPs, which may be attributed to relatively high hydrophobicity of PB-A copolymer. PB-A and PB-B are water insoluble copolymers hence during the step of solvent evaporation, PB-A and PB-B copolymers were precipitated (as DCM will evaporate) and physically entrapped the IgG-Fab in the nanoparticles. However, due to difference in hydrophobicity (PB-A is more hydrophobic than PB-B), PB-A copolymer might have precipitated more rapidly upon exposure to aqueous solutions (W_1 and W_2 phases) than PB-B copolymer. Now slower precipitation of PB-B copolymer may allowed the IgG-Fab to escape from W_1 phase to W_2 phase leading to lower drug loading.

A previous report suggested significant effect of NaCl on EE and DL in nanoparticulate system (Pistel and Kissel, 2000). In Batches 3 and 4, incorporation of NaCl in W_1 (1%) and W_2 (10%) phases exhibited higher EE and IgG-Fab loading in both PB-A NPs (EE = ~55% and DL = 6.68%) and PB-B NPs (EE = ~49% and DL = 5.88%) relative to the respective PB NPs prepared without NaCl. An important prerequisite for higher DL is separation of droplets during emulsification process where organic phase act as a diffusion barrier

between the W_1 and W_2 phases. Higher protein concentration in W_1 phase may elevate osmotic pressure in the internal phase which facilitates diffusion of water from external (W_2) phase. This diffusion may result in thinning of organic phase and eventually lowering EE and DL. Addition of salt in the external phase may help to balance osmotic pressure between W_1 and W_2 phases which resulted in lower diffusion of external phase and higher DL. However, addition of salt did not exhibit substantially enhanced IgG-Fab loading. It may be due to the colligative effects of osmotic pressure. Hence, this effect may enhance loading of lower molecular weight compounds in larger amounts relative to higher molecular weight proteins. Based on these results, PB-A copolymer was selected for further optimization of NP preparation method.

In order to study the effect of drug to polymer ratio, PB-A NPs with two different drug/polymer ratios (1/10 and 1/4) were prepared and evaluated. Results depicted in Batch 5 (Table 2) suggest that as drug/polymer ratio was raised from 1/10 to 1/4, EE was significantly reduced which is in accordance with previously published results (Zhang et al., 2005). This result can be explained by the fact that in case of 1/10 (drug/polymer) ratio, more polymer (10 mg) was available to entrap 1 mg of IgG-Fab relative to 1/4 ratio (drug/polymer). Despite high drug/polymer ratio with Batch 5, there was no significant difference with DL compared to earlier PB-A NPs (Batch 1, Table 2). Such poor DL may be attributed to high volume of external aqueous phase (W_2). Hence, in further studies drug/polymer ratio was kept constant at 1/4 and effect of external phase (W_2) volume was evaluated. For example, in Batch 6 total external phase (W_2) volume was lowered to 5 ml. In addition, double emulsion was prepared with only 2 ml of external aqueous (W_2) phase to reduce protein partitioning in to water (W_2 phase). The resulting multiple emulsion was stabilized by dilution with 3 ml of 2% PVA (W_3 phase). Interestingly, 11.7% of DL was observed with Batch 6 which was significantly higher compared to all earlier batches. Reduction in external phase volume in Batch 6 has significantly improved EE relative to Batch 5.

Based on results from Batch 6, it can be inferred that reduction in external phase (W_2) volume along with high drug/polymer ratio have significant effect on DL. Hence, volumes of all the phases were further reduced while keeping the volume ratio constant. It was hypothesized that the lower volume of W_2 would diminish protein partitioning in aqueous phase improving loading efficiency. In addition, reduction in organic phase volume may raise polymer concentration that may lead to rapid polymer precipitation and NP formation. A DL of 15.61% was observed with NPs in Batch 7 as expected with improved EE (~70%). However, further reduction in volumes (Batch 8) did not result in any improvement in DL (15.47%) or EE (~73%). Optimized process parameters utilized for the preparation of Batch 7 and Batch 8 were denoted as method-A and method-B, respectively.

Process parameters optimized in Batches 7 and 8 for the preparation of IgG-Fab-loaded PB-A NPs were utilized to encapsulate various protein/peptide molecules with different molecular weights such as octreotide (1 kDa), insulin (5.8 kDa), lysozyme (14.7 kDa), IgG (150 kDa) and catalase (237 kDa). As reported in °, the size (molecular weight) of protein/peptide resulted in significant change in DL and EE. Interestingly, peptides/proteins with lower molecular weight i.e., <15 kDa behaved very similarly and exhibited similar EE (35-38%) and DL (8.2-8.8 %) when prepared with either method A or B. Likewise higher

molecular weight proteins (48 kDa) demonstrated similar EE (69.5-78.5%) and DL (15-16.5 %) when prepared with optimized methods A and B. Moreover, no significant differences in EE and DL were noted for insulin, lysozyme, IgG-Fab and catalase loaded PB-A NPs prepared with method A relative to method B. Surprisingly, octreotide (Batch 14) and IgG (Batch 17) loaded PB-A NPs prepared with method B demonstrated lower EE and DL relative to NPs formulated with method A. These results clearly suggest that protein/peptide molecules of certain hydrodynamic diameter or molecular weight behave similarly during NP preparation. Not surprisingly, we have observed effect of molecules and formulation preparation methods on the particle size and polydispersity (Table 3). This may be due to the combined or individual effects of different volumes of W_1 , O and W_2 phases during the NP preparation (Method A & B) and properties of macromolecules. This phenomenon needs to be investigated separately.

$W_1/O/W_2$ methods optimized earlier with PB-A copolymer (Batches 7 and 8) were further utilized for the preparation of NPs with PB-C and PB-D copolymers. As shown in Table 4, mean DL of IgG-Fab in PB-C NPs was 10.49 and 12.49% (Batches 19 and 22). However, loading of IgG-Fab with PB-D copolymer (Table 4) was 17.19 and 16.34% (Batches 25 and 28), which was significantly higher relative to PB-C copolymer. Such higher loading efficiency may be due to the hydrophobic nature of PB-D copolymer. A similar trend in DL was observed for IgG-loaded NPs. There was a small increase in DL with PB-D (Batches 26 and 29) copolymer relative to PB-C copolymer (Batches 20 and 23). With catalase, a 237 kDa protein, DL efficiency remained relatively similar for both PB-C (Batches 21 and 24) and PB-D NPs (Batches 27 and 30) prepared by either method. Overall, as the molecular weight of protein ascends from 48 to 237 kDa, the difference in DL for PB-C and PB-D is diminished. This is possibly to the reduced diffusivity of protein with increased molecular weight.

In vitro release studies—In order to study the release behavior of various proteins, we have performed *in vitro* release studies from a composite formulation comprising PB-A NPs suspended in thermosensitive gel (20 wt%). Figure 6 describes *in vitro* release profiles of octreotide, insulin, lysozyme, IgG-Fab, IgG and catalase from a composite formulation. All the release profiles depicted negligible or no burst release phase. It may be due to the presence of thermosensitive gel which may act as additional diffusion layer for the NP-surface adsorbed protein. Interestingly, we have observed significantly rapid release of octreotide (~93% in 63 days) from composite formulation relative to lysozyme (~72% in 63 days). It may be due to the fact that octreotide has smaller hydrodynamic diameter than lysozyme which may cause facilitated diffusion of octreotide from composite formulation. A similar trend was observed when we compared the release profiles of lysozyme and IgG-Fab (~24% in 65 days). These results clearly suggest that hydrodynamic diameter of protein molecules plays a crucial role in defining release profiles from composite formulation.

However, we have not observed any significant difference between the release profile of octreotide and insulin (~89% in 63 days). It may be possible that difference in molecular weights between octreotide and insulin was not sufficient enough to exert any difference of diffusivity through formulation which may eventually lead to similar release profiles. This fact may also be true for smaller molecular weight proteins. However, we observed no

significant difference between the release profiles of IgG-Fab, IgG (~20% in 65 days) and catalase (~13% in 63 days) from PB-A NPs suspended in thermosensitive gel. It may be possible that IgG-Fab, IgG and catalase possess very low diffusivity through polymer matrix (due to large molecular weights) and hence their release was mainly controlled by degradation of polymers. As published in previous reports degradation of PCL is very slow (Gou et al., 2010, Frank et al., 2005, Huang et al., 2004, Li et al., 2002, Li et al., 2005) and hence it is anticipated that PB copolymers which are comprised of PCL blocks may also degrade slowly. Due to the slower degradation of PB copolymers, release of IgG-Fab, IgG and catalase followed zero order release for a long time period.

Enzymatic activity of lysozyme and catalase

Specific enzymatic activity of lysozyme is reported in Table 5. Lysozyme activity of freshly prepared solution was found to be 61.4 ± 3.8 (U/mg of protein) $\times 10^3$. As described in Table 5, enzyme activity estimated for released samples was relatively higher than the corresponding controls. PB copolymers are composed of PEG, a hydrophilic block. It is anticipated that during NP preparation ($W_1/O/W_2$ double emulsion), PEG may orient at aqueous-organic interface. Hence, PEG may have reduced the interaction of lysozyme with the hydrophobic polymer segments (PCL and PLA) as well as with organic solvent (DCM) which may cause limited denaturation of proteins. However, biological activity of lysozyme was reduced with the respect to time, similar to controls. It may be due to the fact that protein remained in the release medium during a release experiment. Therefore, storage conditions may lower the stability of proteins, which will not be the situation under *in vivo* conditions. Proteins released from composite formulation *in vivo* will be immediately absorbed from the vitreous fluid to the retina.

According to the results depicted in Table 5, enzymatic activity of freshly prepared catalase solution was estimated to be $10.8 \pm 0.17 \times 10^3$. Similar to the lysozyme release samples, enzymatic activity estimated for catalase control samples were relatively lower than the respective release samples. It may be due to the fact that catalase encapsulated into the formulation may have been protected from hydrolytic degradation. Moreover, biological activity of the catalase in release samples and controls were lower due to the storage conditions. The polymer matrix of NPs and thermosensitive gel may have reduced the exposure of proteins to the water molecules and hence protected the lysozymes/catalase from hydrolytic degradation (Singh et al., 2007).

Conclusion

We have successfully optimized the PB nanoparticle preparation methods to achieve high EE and DL for proteins and peptides. During the course, we have observed that PB copolymer with high molecular weight resulted in enhanced EE and DL. Also, addition of salt in internal and external phase had marginal effect of EE, while lowering volume of external phase (W_2) significantly enhanced EE and DL. EE and DL were also found to be directly proportional to molecular weight/hydrodynamic diameter of biotherapeutics. Conversely, *in vitro* release rate was inversely proportional to hydrodynamic diameter/molecular weight of protein/peptides. Moreover, retention of enzymatic activity of lysozyme and catalase in

release samples were also confirmed. These results clearly suggest that PB copolymer based protein-encapsulated formulation may serve as a platform technology for extended delivery of proteins and peptides.

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a**Step 1** **Step 2** **b****Step 2** **Figure 1.**

(a) Synthesis scheme for PB-A and PB-B (PLA-PCL-PEG-PCL-PLA) and (b) synthesis scheme for PB-C and PB-D (PGA-PCL-PEG-PCL-PGA).

Step 1

Step 2

Figure 2.
Synthesis scheme for PB-E (PEG-PCL-PLA-PCL-PEG).

Figure 3.
 ^1H -NMR spectrum of PB-A copolymer in CDCl_3 .

Figure 4.
¹H-NMR spectrum of PB-D copolymer in CDCl₃.

Figure 5.
 $^1\text{H-NMR}$ spectrogram of PB-E copolymer in CDCl_3 .

Figure 6.
In vitro release of protein/peptide from PB-A NPs suspended in PB-E thermosensitive gel (20 wt%).

Table 1

Characterization of various pentablock (PB) polymers.

Code	Structure	Total Mn^a (theoretical)	Total Mn^b (calculated)	Total Mn^c (calculated)	M_w^c (GPC)	PDI ^c
PB-A	PLA ₃₀₀₀ -PCL ₇₀₀₀ -PEG ₂₀₀₀ -PCL ₇₀₀₀ -PLA ₃₀₀₀	22000	20783	17246	24024	1.39
PB-B	PLA ₃₀₀₀ -PCL ₇₀₀₀ -PEG ₄₀₀₀ -PCL ₇₀₀₀ -PLA ₃₀₀₀	24000	21354	17644	23826	1.35
PB-C	PGA ₃₀₀₀ -PCL ₇₀₀₀ -PEG ₄₀₀₀ -PCL ₇₀₀₀ -PGA ₃₀₀₀	24000	21294	16184	23212	1.43
PB-D	PGA ₃₀₀₀ -PCL ₇₀₀₀ -PEG ₂₀₀₀ -PCL ₇₀₀₀ -PGA ₃₀₀₀	22000	20122	17032	24185	1.42
PB-E	PEG ₅₅₀ -PCL ₈₂₅ -PLA ₅₅₀ -PCL ₈₂₅ -PEG ₅₅₀	3300	3275	4330.4	6104.1	1.41

^aTheoretical value, calculated according to the feed ratio

^bCalculated from ¹H-NMR results

^cDetermined by GPC analysis

Optimization of process parameters for the preparation of IgG-Fab-loaded PB NPs. Experiments were performed in triplicates and values are reported in mean $\pm$ standard deviation.

Table 2

Batch #	Process parameters									EE (%)	Drugloading (%)
	Polymer types	Polymer (mg)	IgG-Fab (mg)	Volume of W ₁ (mL)	Volume of organic phase (mL)	Volume of W ₂ (mL) ^a	Volume of W ₃ (mL) ^b	Sonication time W ₁ /O	Sonication time W ₁ /O/W ₂		
Batch 1	PB-A	100	10	1	4	20	-	45 Sec (4 Output)	3 min (7 Output)	54.03 ± 1.80	5.62 ± 0.20
Batch 2	PB-B	100	10	1	4	20	-	45 Sec (4 Output)	3 min (7 Output)	45.72 ± 2.65	4.99 ± 0.29
Batch 3	PB-A	100	10	1 + 1% NaCl	4	20 + 10% NaCl	-	45 Sec (4 Output)	3 min (7 Output)	54.81 ± 2.26	6.68 ± 0.15 [*]
Batch 4	PB-B	100	10	1 + 1% NaCl	4	20 + 10% NaCl	-	45 Sec (4 Output)	3 min (7 Output)	49.49 ± 1.27	5.88 ± 0.19 [*]
Batch 5	PB-A	10	2.5	0.25	1	8.8	-	30 Sec (1 Output)	45 Sec (2 Output)	25.85 ± 2.25 [#]	6.4 ± 1.04 [#]
Batch 6	PB-A	10	2.5	0.25	1	2	3	30 Sec (1 Output)	45 Sec (2 Output)	45.23 ± 1.58 [#]	11.7 ± 1.26 [#]
Batch 7 (Method A)	PB-A	10	2.5	0.167	0.667	1.333	3.667	30 Sec (1 Output)	45 Sec(2 Output)	69.64 ± 9.01 [#]	15.61 ± 1.97 [#]
Batch 8 (Method B)	PB-A	10	2.5	0.1	0.4	0.8	4.2	30 Sec (1 Output)	45 Sec (2 Output)	73.24 ± 0.2	15.47 ± 0.04

^aW₂ phase was comprised of 2% PVA

^bW₃ phase was comprised of 2% PVA

^{*} p < 0.05 (drug loading between batches 1, 2, 3 and 4 are compared)

[#] p < 0.05 (entrainment efficiency and drug loading between batches 1, 5, 6 and 7 are compared)

Encapsulation of various proteins with optimized methods A and B (PB-A). Experiments were performed in triplicates and values are reported in mean +/- standard deviation.

Table 3

Method	Batch #	Protein	EE (%)	Loading (%)	Particle size (nm) / Polydispersity index (PDI)
Method A	Batch 9	Octreotide	35.79 ± 3.63	8.20 ± 0.77	235 / 0.194
	Batch 10	Insulin	38.41 ± 0.75	8.76 ± 0.15	260 / 0.293
	Batch 11	Lysozyme	37.64 ± 1.66	8.60 ± 0.35	340 / 0.306
	Batch 7	IgG-Fab	69.64 ± 9.01 *	15.61 ± 1.97 *	470 / 0.552
	Batch 12	IgG	74.57 ± 1.31 *	15.71 ± 0.23 *	475 / 0.180
	Batch 13	Catalase	78.64 ± 0.81 *	16.43 ± 0.14 *	905 / 0.646
Method B	Batch 14	Octreotide	26.88 ± 1.56	6.30 ± 0.34	220 / 0.178
	Batch 15	Insulin	36.39 ± 2.29	8.34 ± 0.48	235 / 0.188
	Batch 16	Lysozyme	38.44 ± 2.44	8.77 ± 0.51	365 / 0.281
	Batch 8	IgG-Fab	73.24 ± 0.20 *	15.47 ± 0.04 *	645 / 0.530
	Batch 17	IgG	54.56 ± 2.56 *	12.00 ± 0.49 *	605 / 0.658
	Batch 18	Catalase	76.87 ± 0.58 *	16.12 ± 0.10 *	810 / 0.221

* p < 0.05 (drug loading and entrapment efficiency of batches 7, 12, 13, 8, 17, and 18 are compared with batches 9, 10, 11, 14, 15 and 16).

Table 4

Encapsulation of various proteins with optimized methods A and B using two different PB polymers (PB-C & D). Experiments were performed in triplicates and values are reported in mean $\pm$ standard deviation.

Polymer	Method	Batch #	Protein	EE (%)	Loading (%)
PB-C	Method A	Batch 19	IgG-Fab (48 kDa)	46.87 $\pm$ 0.84	10.49 $\pm$ 0.17
		Batch 20	IgG (149 kDa)	72.02 $\pm$ 0.71 *	15.26 $\pm$ 0.13 *
		Batch 21	Catalase (237 kDa)	75.84 $\pm$ 1.19 *	15.94 $\pm$ 0.21 *
	Method B	Batch 22	IgG-Fab (48 kDa)	57.15 $\pm$ 8.49	12.49 $\pm$ 1.62
		Batch 23	IgG (149 kDa)	68.54 $\pm$ 1.42 *	14.63 $\pm$ 0.26 *
		Batch 24	Catalase (237 kDa)	72.08 $\pm$ 0.68 *	15.27 $\pm$ 0.12 *
PB-D	Method A	Batch 25	IgG-Fab (48 kDa)	83.09 $\pm$ 1.44 *	17.19 $\pm$ 0.25 *
		Batch 26	IgG (149 kDa)	85.31 $\pm$ 0.4 *	17.58 $\pm$ 0.07 *
		Batch 27	Catalase (237 kDa)	72.89 $\pm$ 1.21 *	15.41 $\pm$ 0.22 *
	Method B	Batch 28	IgG-Fab (48 kDa)	78.09 $\pm$ 0.44	16.34 $\pm$ 0.08
		Batch 29	IgG (149 kDa)	81.77 $\pm$ 0.63	16.97 $\pm$ 0.11
		Batch 30	Catalase (237 kDa)	76.89 $\pm$ 1.18	16.12 $\pm$ 0.21

* p < 0.05 (drug loading and entrapment efficiency of batches 19 and 22 compared with batches 20, 21, 23 and 24. Similarly, drug loading and entrapment efficiency of batches 25, 26 and 27 are compared with batches 28, 29 and 30).

Enzymatic activity of lysozyme and catalase estimated in the *in vitro* released samples. Experiments were performed in triplicates and values are reported in mean $\pm$ standard deviation.

Table 5

Specific enzyme activity of Lysozyme (U/mg) $\times 10^3$			Specific enzyme activity of Catalase (U/mg) $\times 10^3$		
Time (days)	Release samples	Controls	Time (days)	Release samples	Controls
4	61.2 $\pm$ 3.4	59.5 $\pm$ 1.7	1	10.79 $\pm$ 1.17	10.59 $\pm$ 0.48
15	53.3 $\pm$ 4.2	46.8 $\pm$ 4.4	15	5.47 $\pm$ 0.13	5.04 $\pm$ 0.19
45	35.7 $\pm$ 2.1	26.7 $\pm$ 2.7	45	3.88 $\pm$ 0.16	3.59 $\pm$ 0.20
63	26.3 $\pm$ 3.6	18.9 $\pm$ 1.6	63	3.63 $\pm$ 0.20	3.26 $\pm$ 0.31