DEVELOPMENT OF PENTABLOCK COPOLYMER BASED FORMULATIONS FOR THE SUSTAINED DELIVERY OF PROTEIN THERAPEUTICS IN THE TREATMENT OF POSTERIOR SEGMENT OCULAR DISEASES

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We have successfully synthesized pentablock (PB) copolymers comprised of various FDA approved polymer blocks such as polyethylene glycol (PEG), polycaprolactone (PCL), polylactic acid (PLA) and polyglycolic acid (PGA). PB copolymers with different composition, molecular weights and block arrangements were utilized to develop protein-embedded thermosensitive gels or nanoparticles (NPs) for sustained delivery in the treatment of posterior segment ocular diseases. In order to eliminate the burst release effect, we have studied PB composite formulation comprised of protein-encapsulated PB NPs dispersed in PB thermosensitive gel. The composite formulation eliminated burst release effect and exhibited nearly zero-order protein release for significantly longer durations. In this research work, we have utilized various model proteins (lysozyme, IgG-Fab, IgG, BSA, and catalase) and therapeutic proteins (octreotide, insulin and bevacizumab) to optimize the formulation.

We have synthesized various triblock (TB) (PCL-PEG-PCL, B-A-B) and PB (PLA-PCL-PEG-PCL-PLA (C-B-A-B-C) and PEG-PCL-PLA-PCL-PEG (A-B-C-B-A)) copolymers based thermosensitive gelling polymers. We have observed distinct effect of block arrangement and molecular weights of block copolymers on the sol-gel transition and on the kinematic viscosity of aqueous solutions. PB copolymers with A-B-C-B-A block arrangement exhibited significantly lower viscosity relative to TB copolymers or other types of PB copolymers (C-B-A-B-C). The difference in viscosity and sol-gel transition behavior has been
explained by two different processes of micellization for A-B-C-B-A and B-A-B, or C-B-A-B-C types of copolymers. Moreover, a PB copolymer based formulation sustained the release of IgG up to ~20 days, which is significantly longer relative to TB copolymers based formulations.

In order to sustain release for longer duration, we have synthesized various PB copolymers (PLA-PCL-PEG-PCL-PLA and PGA-PCL-PEG-PCL-PGA) with high molecular weight and utilized them for the fabrication of protein-encapsulated NPs. We observed a significant effect of the presence of PLA or PGA on entrapment efficiency (EE), drug loading (DL) and in vitro release behavior. This may be due to the fact that PB copolymers exhibited significantly reduced crystallinity relative to TB copolymers. In addition, we have successfully optimized NP preparation methods to achieve maximum possible DL. This achievement allowed the loading of a large amount of drug which can last for ~6 month in a limited injection volume (100 µL). The optimized methods were successfully utilized to encapsulate a wide variety of peptides and proteins with molecular weights ranging from 1 - 237 kDa in PB NPs. PB NPs alone exhibited significant burst release in the first few days of release study. However, a composite formulation comprised of protein-encapsulated PB-NPs prepared with optimized method and optimized PB copolymers (PB copolymers for NPs and thermosensitive gel) exhibited protein release for significantly longer duration of time (~6 months) with nearly zero-order release rate.

We have evaluated the structural integrity of released protein at different time intervals by CD spectroscopy. Moreover, biological activity of bevacizumab was evaluated by cell proliferation and cell migration assays. Enzymatic activity of lysozyme and catalase were confirmed with their respective enzymatic assays. Our results indicated that proteins retained
their structural integrity and bioactivity during the preparation of formulation and also during the release process. *In vitro* cell culture studies such as cell viability, cytotoxicity and biocompatibility studies performed on various ocular cell lines confirmed the safety of PB copolymers for ocular applications. Further, we have performed *in vivo* ocular tolerability studies with optimized PB formulations which demonstrated no inflammation, retinal toxicity, change in intraocular pressure or cataract even after 16 week of exposure. Moreover, *in vivo* studies further revealed that PB copolymers based formulations were slowly degraded and dissolved in vitreous humor confirming biodegradability of polymers.

Our studies indicated that PB copolymer based composite formulation can serve as a platform technology for the development of sustained release therapy in the treatment of posterior segment ocular diseases such as wet age-related macular degeneration (wet-AMD), diabetic macular edema (DME) and diabetic retinopathy (DR). This technology has a scope beyond ocular treatments and can also be used for the treatment of other chronic diseases.
The faculty listed below, appointed by the Dean of the School of Graduate Studies have examined a dissertation titled “Development of Pentablock Copolymer Based Formulations for the Sustained Delivery of Protein Therapeutics in the Treatment of Posterior Segment Ocular Diseases” presented by Sulabh P. Patel, candidate for the Doctor of Philosophy degree, and certify that in their opinion it is worthy of acceptance.

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CHAPTER 1

LITERATURE REVIEW

Development of controlled and sustained ophthalmic delivery systems still remains a major area for pharmaceutical research. It is even more relevant today because of the emergence of new and potent drugs, especially biological modifiers. Recent advances in the field of nanotechnology have led to the development of novel drug delivery systems for ocular complications.

Ocular barriers, routes of administration and their significance in drug delivery [1]

Various routes of administration have been explored for the local and targeted delivery of the therapeutics in the treatment of ocular diseases (Figure 1.1, anatomical locations for various routes of administration denoted in bold & italic). An understanding of various ocular barriers will be necessary to design novel drug delivery systems for the treatment of ocular diseases. Topical ophthalmic delivery systems are indicated for the treatment of anterior chamber diseases including glaucoma, allergic conjunctivitis, corneal epithelial keratitis, stromal keratitis and dry eye. For most topical administration, the sites of action are different anterior chamber tissues such as the layers of cornea, lacrimal glands, sclera, conjunctiva, iris and ciliary body. Topical formulations are also prescribed for the treatment of back of the eye diseases such as diabetic retinopathy (DR), bacterial endophthalmitis, age-related macular degeneration (AMD) and retinitis. However, the physical barriers imposed by various structural defensive mechanisms of the eye lead to poor absorption of the therapeutics after topical administration [2].
Figure 1.1: Anatomical sites for various routes of ocular drug delivery [1].
Figure 1.2: Corneal barriers [2]
The main limitations for conventional topical drug delivery are higher tear turnover rate and lower precorneal residence time, which lead to poor drug concentration or subtherapeutic levels in the anterior chamber, requiring frequent administration [3].

In addition, different layers of cornea, sclera and conjunctiva play a vital role in drug transport after topical administrations. The cornea, the outermost layer of the eye, can be divided into three distinct layers, epithelium, stroma, and endothelium (Figure 1.2). The corneal epithelial cells are highly lipoidal and are attached to each other by desmosomes and zonula occludens (tight junction complexes) [4, 5]. Due to their lipoidal nature, these cells act as a mechanical barrier for hydrophilic drugs. Moreover, the presence of tight junctions offers significant resistance to paracellular transport of various hydrophilic and large molecules. The corneal epithelium is followed by stroma, which comprises 90% of corneal thickness. Stroma is a highly hydrated lamellar arrangement of collagen fibers, and it acts as a static barrier for hydrophobic drugs. Corneal endothelial cells are layered between the aqueous humor and stroma. The tight junctions of these cells are leaky and facilitate the permeability of macromolecules between the stroma and aqueous humor [6].

Higher blood and lymphatic flow in conjunctiva eliminate a large fraction of the administered dose into systemic circulation and thus is considered as non-productive absorption. Moreover, tight junction complexes of the conjunctival epithelium prevent passive diffusion of hydrophilic drugs [7]. The sclera is a continuous layer, which originates from the limbus and extends posteriorly throughout the eye globe. It is a hydrophilic tissue composed of proteoglycans and collagen fibers. Scleral permeability of the therapeutics is inversely proportional to the hydrodynamic radius of the permeating molecules. For example, globular proteins were more permeable relative to dextrans with linear structures [8]. Furthermore,
permeability across the sclera is also affected by charge. A recent report suggests that permeability of anionic molecules is higher compared to cationic molecules, and it might be due to the interaction between positively charged molecules with the negatively charged proteoglycans [9].

Although intravitreal and periocular administrations are not patient compliant, these routes are employed to deliver therapeutics for the treatment of posterior segment diseases. The periocular route is relatively less invasive than the intravitreal route, which includes subtenon, subconjunctival, peribulbar and retrobulbar administrations (Figure 1.1). After periocular administrations, drug molecules can reach back of the eye by three different routes: systemic circulation through the choroid, transscleral pathway, and the anterior route (tear film, cornea, aqueous humor and vitreous humor) [10]. Subconjunctival administration can bypass conjunctival epithelium, which is the rate limiting factor for the absorption of hydrophilic drugs. However, many metabolic, static and dynamic barriers (conjunctival blood and lymphatic circulations) increase the elimination of the drug and eventually, reduce drug transport to the posterior section [11, 12]. Molecules, which escape the conjunctival vasculature, eventually reach to the photoreceptor cells and the retina via sclera and choroid. Permeability across the sclera is not a major constraint because it is highly dependent upon the molecular radius and not the hydrophobicity of drug molecule [13, 14]. However, choroidal blood circulation does act as a dynamic barrier for the drug permeability and eliminates a considerable amount of the dose. Nevertheless, the blood-retinal barrier (BRB) also plays a critical role for permeability of active molecules to the photoreceptor cells (Figure 1.3). Direct delivery of therapeutics to the vitreous humor can offer enormous advantage over the subconjunctival administration.
Figure 1.3: Blood retinal-barriers [2]
However, intravitreal injection has the least patient compliance and it also leads to non-uniform drug distribution in the vitreous humor. The vitreous allows rapid diffusion to small molecule, while it restricts the diffusion of macromolecules. Furthermore, pathophysiological conditions also have a significant effect on the distribution of therapeutics [15].

**Role of cell membrane transporters and melanin in drug ocular bioavailability**

The roles of transporters in ophthalmic drug delivery have been reviewed by many investigators [16-18]. Transporters play an important role in the ocular bioavailability of drug molecules. There are two types of transporters present on the ocular tissues: influx and efflux transporters. Out of these two transporters, the efflux transporter acts as a barrier for the drug absorption. Polyglycoprotein (P-gp), multi drug resistance protein (MRP) and breast cancer resistance protein (BCRP) have been identified on different ocular tissues and cell lines. In case of *in vitro* cell models, transporter protein expression pattern may vary according to its culture conditions and origin [19, 20]. P-gp has significant affinity for lipophilic drug molecules. It has been identified on conjunctiva [21, 22] and retinal pigment epithelium (RPE) [19, 23, 24]. However, recent reports indicate that functional expression of P-gp on human corneal epithelium may be absent or negligible [20, 25]. Out of nine isoforms of MRP, only three have been identified on various ocular tissues. Furthermore, presence of MRP1 has been reported on RPE [26]. The presence of BCRP on corneal epithelium cells has also been reported [27]. Freshly excised human corneal epithelial tissue showed the functional expression of MRP1, MRP5, and BCRP [20].

Melanin is located in various ocular tissues such as uvea, RPE, and choroid. It has a tendency to interact with lipophilic and basic drug molecules *via* van der Waals and electrostatic forces or by simple charge transfer [28, 29]. Binding of melanin to therapeutics
can significantly reduce the pharmacological activity [30]. In the anterior chamber, binding of
drug molecules with melanin renders them unavailable for the receptor mediated uptake which
eventually reduces ocular bioavailability [31] and necessitates the administration of larger dose
[32]. Likewise, systemic or transscleral drug administration may be followed by interaction of
drug molecule with melanin in RPE or choroid, which can reduce the fraction of molecules
permeating to retina and vitreous humor [33, 34]. Higher binding of lipophilic drugs to the
melanin was demonstrated in bovine choroid-Bruch’s membrane [35].

A current challenge in ophthalmic drug delivery is to develop a tissue targeted delivery
system, which can circumvent ocular barriers without altering ocular protective mechanisms.
Colloidal or particulate drug delivery systems can evade many static, dynamic, and metabolic
barriers. Moreover, they can offer the simplicity of a topical eye formulation and it can also be
utilized for intraocular and periocular injections.

**Various strategies for ocular drug delivery**

*Additives* [36]

Several attempts have been made to improve drug availability into the anterior chamber
either by utilizing enhancers to increase drug permeability across ocular tissues or by
employing mucoadhesives to enhance precorneal residence time of the applied dose.

Viscosity enhancing polymers can be incorporated into ophthalmic formulations to
improve residence time in the precorneal area and to increase absorption of the therapeutic
agents into and across the cornea. Various polymers, such as hydroxypropylmethylcellulose
(HPMC) [37], hyaluronic acid (HA) [38, 39], polyvinyl alcohol (PVA) [37],
hydroxyethylcellulose [37], and methylcellulose [40] have been investigated to determine their
potential in improving the bioavailability of therapeutic agents following topical applications.
**Pluronics**

Pluronics can significantly improve drug solubility and enhance the viscosity of topical formulations. Pluronic-F68 (15%) and Pluronic-F127 (10%) were more effective as an indomethacin solubilizer and viscosity enhancer relative to polyols and polysorbate 80 [41]. Moreover, Pluronic solutions were well tolerated on rabbit eyes than commercial formulation, Indocid-suspension. Poloxamer-407 [polyoxyethylene- polyoxypropylene block copolymer] was evaluated as a solubilizer for topical application of indomethacin [42]. This formulation has significantly elevated indomethacin levels in aqueous humor (AUC = ~21 h.µg/mL) relative to the marketed solution (AUC = ~8 h.µg/mL). In addition, anti-inflammatory studies performed on an immunogenic uveitis model demonstrated comparatively more rapid resolution of the symptoms. In another study, PVA and HPMC caused improvement in viscosity and stability of indomethacin topical ocular suspension [43]. Mucoadhesive eye drops of tolmetin (a pyrrole-acetic acid derivative) exhibited higher drug levels in aqueous humor in comparison to the aqueous solution, in both inflamed and uninflamed rabbit eyes [44].

A novel approach of a drug-embedded thermosensitive gel demonstrated some promising results for topical ophthalmic drug delivery. In a recently published study Gao, et al. have prepared and evaluated a dexamethasone-loaded polylactide-co-glycolide-PEG-polylactide-co-glycolide (PLGA-PEG-PLGA) thermosensitive gelling solution [45]. This formulation improved precorneal residence time and demonstrated 7-fold higher drug levels in aqueous humor (C<sub>max</sub> = 125.2 mg/mL) relative to eye drops. This hydrogel system showed good biodegradability, sustained release, and ocular biocompatibility, indicating that the system is a safe candidate for sustained ophthalmic drug delivery. Many other in situ polymeric
gelling systems, such as chitosan [46], poloxamer [47], HPMC [48], PEG-PCL-PEG [49], poly N-isopropylacrylamide/chitosan [50], poloxamer/chitosan [51], pluronic F-127/chitosan [52], and poloxamer/carbopol [53] were explored for ocular applications.

_Cyclodextrins_

One of the strategies to enhance the ocular absorption of poorly soluble drugs is by increasing their aqueous solubility. Almost all NSAIDs and corticosteroids are highly lipophilic molecules, which exhibit very poor aqueous solubility, and therefore it is very challenging to formulate them in aqueous eye drops. Cyclodextrins have been commonly used to enhance the solubility of various hydrophobic drugs, owning to their excellent drug solubilizing property. Drug solubilizing property of cyclodextrins is highly dependent on their ability to form water soluble complexes with drug molecules. Such complexes interact with the membrane wherein, the hydrophobic drug molecules diffuse through the lipid membrane while the water soluble cyclodextrin molecules are retained in the aqueous tear fluid. It has been reported that the amount of cyclodextrin required to solubilize lipophilic drugs should be just enough (<15%) for improving drug penetration following topical administrations. A higher concentration of cyclodextrin retains the drug in the aqueous tear fluid, thereby decreasing its transcorneal penetration. The release rate of drug molecules from these complexes along with the partition of the drug into and then through the corneal and conjunctival epithelium determines the intraocular availability of the drug [54].

In a recently published manuscript Valls, et al. demonstrated improved solubility and a higher ocular bioavailability of diclofenac [55]. Six times higher transport of diclofenac across the corneal tissue was observed with β-CD/diclofenac complex treatment relative to free drug. Loftsson, et al. have examined the effects of various CDs (randomly methylated β-CD
(RMβCD) and 2-hydroxypropyl-β CD (HPβCD)) on ocular delivery of dexamethasone [56].

Results from in vivo ocular tissue distribution studies illustrated that both lipophilic RMβCD and hydrophilic HPβCD have improved dexamethasone levels in rabbit eyes. However, RMβCD delivered higher amounts of dexamethasone relative to other CDs.

The same investigators have recently published a patent, demonstrating the role of CDs (RMβCD and γ-cyclodextrin (γ-CD)) in the delivery of corticosteroids to various ocular tissues [57]. Nanoparticulate formulation of γ-CD-drug conjugates were able to deliver corticosteroids more efficiently to the back of the eye, contrary to the RMβCD drug solution, which causes localization of more drug into the anterior chamber of the rabbit eyes. Cyclooxygenase-2 (COX-2) inhibitors are also indicated in the treatment of ocular inflammations. However, poor aqueous solubility of these agents limits their topical application. In an attempt to improve ocular bioavailability, a nanoparticulate formulation of valdecoxib with HPβCD was evaluated [58]. As anticipated, levels of valdecoxib in the cornea and conjunctiva were significantly higher in NP-treated rabbit eyes relative to control.

In vivo ocular bioavailability of three different hydrocortisone (HC) formulations (1% HC solution with HPβCD, 1% HC solution with HPβCD along with sodium hyaluronate or carbopol 934P, and 1% HC suspension) was evaluated in New Zealand White rabbit [59]. Incorporation of HPβCD in formulation improved HC solubility and bioavailability in the cornea and aqueous humor by 75% and 55%, respectively, relative to aqueous suspension. Interestingly, inclusion of hyaluronate or carbopol in the HPβCD solution did not alter ocular bioavailability.

In clinical trials, topical delivery of the dexamethasone/HPβCD solution exhibited a significantly higher aqueous humor concentration (2.6-fold higher AUC) in human subjects.
relative to suspension [60]. Currently, several CD containing formulations are marketed in Europe, such as Indocid (indomethacin with HPβCD) and Voltaren (diclofenac-Na with HPβCD), for the treatment of anterior chamber inflammations. It will not be surprising if CD containing ophthalmic topical formulations enter the US market in the near future.

**Colloidal dosage forms for drug delivery to anterior segment of the eye**

In the last decade, many novel strategies including liposomes, polymeric NPs, dendrimers, nanoemulsions, micelles, nanosuspensions, and combination approaches have been investigated for the development of sustained ocular drug delivery systems. These colloidal dosage forms offer numerous advantages over conventional dosage forms, such as higher drug solubility, enhanced bioavailability, improved physical and chemical stability, and sustained drug delivery. Furthermore, nanocarriers can lower toxicity and irritability concerns related to drug and/or formulation, and eventually these systems can improve *in vivo* performance and patient compliance. Such delivery systems can significantly bypass the blood-ocular barriers, overcome the efflux related issues of parent drugs, and reduce frequency of administration [61]. However, a clear understanding of the size, charge, and affinity of drug molecules towards various ocular tissues and pigments are crucial for the development of effective ocular formulations. For example, for transcorneal delivery, it is important to understand the structure and properties of each layer of the cornea. Corneal epithelium facilitates the transport of hydrophobic drugs, while the stroma acts as a barrier for hydrophobic drugs and allows only hydrophilic drugs to enter into the anterior chamber. Corneal mucosa is negatively charged, and therefore it increases the permeability of positively charged drugs and prolongs retention of positively charged nanocarriers [62]. Other than the transcorneal route, conjunctival and transscleral routes play a vital role for the treatment of
posterior segment diseases. Permeability of a drug molecule across the sclera depends upon various physicochemical properties such as hydrodynamic radius, molecular weight, surface charge, and hydrophilicity. Studies revealed that 200 nm particles could not cross the sclera, while 20 nm particles crossed the scleral tissue at very low extent [63, 64]. Similarly, the performance of nanocarriers is also affected by choroidal circulations (dynamic barrier: blood and lymphatic flow), surface modifications with endogenous molecules, size, and abundance of various enzymes particularly for biodegradable nanocarriers.

**Liposomes**

Research on liposomes has expanded considerably in the last thirty years. Liposomes are spherical biphasic vesicular system where the internal aqueous phase is surrounded by phospholipid bilayer membrane [65]. Hydrophilic drugs can be encapsulated in the inner aqueous core while the hydrophobic drugs tend to stay in the lipid bilayer [66]. Depending on the size, liposomes can be classified into small unilamellar vesicles (SUV) (10-100 nm), large unilamellar vesicles (LUV) (100-300 nm) and multilamellar vesicles (contains more than one bilayer). Liposomes can be formulated from sphingolipids, long chain fatty acids, cholesterols, glycolipids, membrane proteins, and non-toxic surfactants [67]. Therapeutic molecules such as proteins, nucleotides, small molecules, and even plasmids can be delivered with liposomes [68]. These delivery systems can be employed to control and sustain the release of therapeutic molecules, and more importantly, they can be used to protect the therapeutic agent from metabolic degradation [69]. Due to numerous advantages, liposomes have been extensively investigated in ophthalmic treatments.

Investigators have studied transcorneal permeation of neutral, anionic and cationic liposomes. Cationic liposomes interact more efficiently with negatively charged corneal
epithelial membrane relative to the anionic or neutral liposomes, and eventually provide higher
drug (tropicamide, penicillin G and acetazolamide) transport across the cornea [70-72]. Along
with small molecule drug delivery, liposomes have also been investigated as non-viral vectors
for gene transfections [73] and as a carrier for the delivery of monoclonal antibodies [74].
Recently, a focal laser was employed to manage the release of drug and dyes from liposomes
at the target site [75]. In the future, laser-targeted delivery system may be employed for the
treatment of neovascular vessel occlusion, choroidal and retinal blood vessel stasis,
angiography, and selective tumors.

Liposomes prepared with naturally derived phospholipids such as egg phosphatidyl
ethanolamine or dioleoyl phosphatidyl ethanolamine (DOPE) are more suitable for the
ophthalmic drug delivery purposes [76]. Liposomes have been successfully utilized for drug
delivery to the anterior segment of the eye. The therapeutic efficiency of liposomes depends
on various factors, including size and charge, encapsulation efficiency, retention and stability
in conjunctival sac and ocular tissues and affinity towards corneal surface. The surface charge
of liposomes plays a key role in determining their affinity towards the corneal surface.
According to Felt, et al. negatively charged corneal surface has higher affinity towards
positively charged liposomes. They also showed that the drug elimination due to lachrymal
flow was reduced as the cationic liposomes increased the viscosity and interacted with the
negatively charged mucus [77]. Liposomes-loaded with pilocarpine hydrochloride were
prepared by Monem, et al. and researchers observed that neutral MLVs showed the most
prolonged effect when compared to negatively charged MLVs and free drug [78]. Acyclovir
(ACV) liposomes were formulated and evaluated for their in vitro permeation and in vivo
absorption across the cornea in rabbits. Experiments showed that positively charged liposomes
formed a coating on the corneal surface. The morphology depicted that liposomes bound tightly to the corneal tissue and increased the residence time and thus improved the ACV absorption [79]. Ocular pharmacokinetics of ganciclovir (GCV) encapsulated in liposomes was studied in albino rats and compared with GCV solution. Transcorneal permeability of GCV liposomes was 3.9 fold higher when compared to GCV solution. The AUC of GCV in aqueous humor was found to be 1.7 fold higher in case of GCV liposomes. Ocular tissue distribution of GCV from liposomes demonstrated 2-10 times higher concentration in sclera, cornea, iris, lens and vitreous humor when compared to solution treated groups. These results suggest that liposomes can efficiently deliver GCV to the eye [80]. Ciprofloxacin-loaded liposomes suspended in hydrogels were formulated using two phospholipids, soya bean phosphatidyl choline and cholesterol. The encapsulation efficiency of drug was found to be 82 ± 1%. Transcorneal permeation profiles of ciprofloxacin from 0.3% aqueous solution, 0.3% liposomal suspension and 0.3% liposomal hydrogels were studied for 6 h. The cumulative drug permeated across cornea was 201 ± 9 µg with aqueous solution and the percentage permeation was 6.7%; while the cumulative amount permeated from liposomal suspension was 614 ± 14 µg and percentage permeation was 20.4% and with liposomal hydrogel formulation, ciprofloxacin permeation was 918 ± 25 µg and the percentage permeation was 30.6%. Investigators have observed that the liposomal suspension exhibit three-fold increase in permeation than aqueous solution [81]. This may be mainly attributed to the electrostatic interaction between positively charged liposomes and the negatively charged corneal membrane. The liposomes are well adsorbed onto the corneal surface and transfer their membrane associated drug directly into the corneal epithelial cell membranes, thereby enhancing drug transport across cornea [82]. Similarly, liposomes of ofloxacin and gatifloxacin
were prepared and studied for determining their efficiency in increasing the drug ocular bioavailability [83]. In case of ofloxacin liposomal hydrogel the permeation was seven fold higher than that of aqueous solution. Hence liposomal hydrogels can overcome all the precorneal barriers and ensures steady and prolonged transcorneal permeation of the drug.

Like other delivery systems, liposomal treatment is also associated with few drawbacks, such as possible toxicity and irritability [84-87]. Lipid components of the liposomes are believed to be a primary source of toxicity, while charge of the liposomes is a main reason for irritability. These constraints may restrict their chances for becoming popular ophthalmic dosage form of the future. Moreover, commercial success of liposomes is also limited because of the difficulties in sterilization and their relatively short shelf life.

**Polymeric NPs**

In order to address the problems of possible toxicity and irritability associated with liposomes, drug-loaded nanometer sized polymeric particles may be considered a viable alternative for the development of sustained release ophthalmic formulation. Nanoparticulate systems comprise of particles with less than one micron particle size in which therapeutically active agent is entrapped, absorbed, encapsulated, attached or adsorbed [67]. Aqueous or non-aqueous suspension of drug-loaded polymeric NPs can either be delivered as topical drops in the cul-de-sac or can be administered via transscleral, intravitreal or intraperitoneal routes. Development of sustained release biodegradable dosage form for intravitreal delivery may evade the limitation of frequent administration and it may also improve patient compliance. Polymeric NPs can sustain drug release by diffusion, dissolution, or mechanical disintegration and/or erosion of the polymer matrix [88]. NPs can circumvent the limitation of poor solubility of therapeutics. Moreover, the NP can also protect the drug (e.g. peptides and proteins) from
enzymatic degradation, and eventually, improves an ocular bioavailability. Thus, NPs are a better candidate for both posterior and anterior segment delivery.

Various biodegradable and non-biodegradable polymeric systems have been developed for sustained delivery of NSAIDs, such as ibuprofen, flurbiprofen, and indomethacin in the treatment of anterior chamber inflammations. In a recently published study, investigators have utilized Eudragit RS100 to prepare ibuprofen NPs for inhibition of an inflammatory response to surgical trauma [89]. Results from in vivo efficacy studies performed on the rabbit eye model demonstrated a significantly higher aqueous humor concentration than the control aqueous eye drop. Similar studies were performed with flurbiprofen as an active agent for the prevention of myosis induced by extracapsular cataract surgery [90]. A higher interaction of positively charged NPs (zeta potential +40–60 mV) with an anionic corneal surface was observed [91]. A higher precorneal retention achieved with controlled release formulation was noted to be a primary reason for improvement in flurbiprofen ocular bioavailability. Ocular applications of indomethacin are overshadowed by its poor availability. In an attempt to improve ocular bioavailability Calvo, et al. have examined three different colloidal carrier systems, that is, NPs, nanocapsules, and nanoemulsions [92]. Results of ex vivo transport studies across the excised rabbit cornea demonstrated higher indomethacin ocular bioavailability, due to the colloidal nature of the carrier system. Two different biodegradable polymers (PLGA and PCL) were utilized to formulate flurbiprofen-encapsulated NPs to improve ocular availability [55]. Significantly enhanced corneal transport of flurbiprofen was observed in case of a nanocarrier system relative to free drug. Moreover, PLGA NPs demonstrated ~2-fold higher transport of flurbiprofen compared with the PCL NPs. Subsequently, flurbiprofen-loaded PLGA NPs were prepared and evaluated by Vega, et al. [93]. Incorporation of Poloxamer 188 in NP preparation
has significantly improved stability of NPs. Moreover, topical instillation of NP formulation in the rabbit eyes, enhanced anti-inflammatory efficacy without any signs of irritation or toxicity to ocular tissues. Improved efficacy could be due to an improvement in the bioadhesive property of NPs.

Cyclosporin-A (CS-A)-loaded chitosan NPs were successfully prepared and evaluated for topical ocular applications [94]. Significantly positive zeta potential and a smaller particle size improved precorneal retention of NPs. In vivo studies have revealed that topical instillation of chitosan NPs can selectively increase CS-A levels in the cornea (2-fold higher) and in the conjunctiva (~4-fold higher) relative to topical eye drops of the chitosan solution or aqueous suspension of CS-A. Cholesterol-conjugated hydrophobically modified chitosan was utilized to prepare CS-A-encapsulated NPs [95]. Higher NP retention at the precorneal surface was confirmed by single photon emission computed tomography and scintillation counter measurement. In another study, the same research group has disclosed physical mixture of PLA/chitosan to prepare rapamycin-loaded NPs [96]. Incorporation of PLA significantly improved NP encapsulation efficiency (~13-fold) due to stronger hydrophobic interactions. In vivo studies were conducted in rabbits with topical dosing of rapamycin-loaded NPs, empty NPs, and no treatment. Post-treatment inflammation or blood vessel development was monitored. Results for treatment with rapamycin-loaded NPs demonstrated clear and transparent corneas. On the contrary, corneas, which received no treatment or empty NP treatment were found opaque with stromal edema and/or neovascularization, within first 10 days. In addition, the rapamycin suspension exhibited some degree of inhibitory effect on neovascularization.
Prednisolone is one of the most effective agents in a group of glucocorticoids, and it is marketed as ocular suspensions and drops. This product inhibits a wide variety of inflammatory responses, such as fibrin disposition, leukocyte migration, fibroblast proliferation, edema, capillary dilation, and capillary proliferation. Gatifloxacin and prednisolone were simultaneously incorporated in mucoadhesive polymer (HA)-coated Eudragit NPs (RS 100 and RL 100) in the treatment of bacterial keratitis [97]. Noticeably, improved ocular bioavailability (corneal and aqueous humor) for gatifloxacin was observed after topical instillation of NP suspension. However, investigators did not evaluate ocular tissue distribution of prednisolone.

Recently Alonso, et al. have patented CS-A and indomethacin-encapsulated PCL NPs for ocular drug delivery [98]. Specific ingredients such as chitosan and lecithin have provided positive charge to NPs and also improved stability of the formulation. Recently, Mitra and Mishra developed PB copolymer system with both NPs forming and thermosensitive gelling ability with changing the polymer block ratio [99]. These polymeric systems may be employed for treatment of chronic anterior ocular diseases.

Role of CD44 HA receptors, located on human corneal and conjunctival cells, in the uptake of hyaluronic acid-chitosan oligomer based NPs (HA-CSO NPs) have been studied. Results demonstrated that plasmid-loaded HA-CSO NPs undergo active transport mediated by CD44 HA receptors via caveolin-dependent endocytosis pathway [100]. Confocal studies demonstrated involvement of CD44 HA receptors mediated fluidic endocytosis, internalizing the plasmid-encapsulated HA-chitosan NPs [101]. Similarly Enriquez de Salamanca, et al. documented the contribution of active transport mechanism for internalization of chitosan NPs by human conjunctival epithelial cells [102]. Current research is focused on utilizing various transporters or receptors expressed on the cell surface for active targeting. Targeting specific
transporters or receptors with functionalized NPs may facilitate enhanced uptake into ocular tissues. Kompella, et al. studied the effect of surface functionalization on the uptake of NPs employing ex vivo bovine eye model [103]. NPs surface functionalized with deslorelin, a luteinizing hormone-releasing hormone agonist, or transferrin demonstrated 64% and 74% higher transport respectively, relative to non-functionalized NPs.

**Nanosuspension**

It is very difficult to formulate a poorly soluble drug in conventional ophthalmic dosage forms. Many approaches have been explored to enhance the solubility of such drugs to make them suitable for the preparation of ophthalmic formulations. A general approach for enhancing the solubility of drugs is micronization wherein the drug particle size is reduced to approximately 0.1 mm to 25 µm. However, this size range is not sufficient to increase the saturation solubility of the drug and hence its ocular bioavailability. Use of co-solvents can also improve drug solubility but they are not devoid of toxic effects. The most commonly used approach to trim down drug solubility issues is to formulate the drug into nanosuspension. Nanosuspension is a colloidal dispersion of nanosized particles. Nanosuspensions can be prepared by precipitation, pearl milling and high pressure homogenization techniques. Moreover, the solid state of the drug in nanosuspensions minimizes the problem of chemical stability of the drug as well as the physical stability of the formulation [104]. For ocular drug delivery, nanosuspension provides numerous advantages such as dose reduction, ease of eye drop formulation, increased bioadhesion and corneal penetration, reduced ocular irritation and enhanced bioavailability.

Nanosuspension is stabilized by other excipients, such as surfactants, viscosity enhancers, or charge modifiers. Topical delivery of 1% aqueous suspension and 1% oil
suspension in human subjects demonstrated higher levels of indomethacin in aqueous humor relative to oral delivery [105]. Notably, oil suspension exhibited higher aqueous humor levels (429 ng/mL) relative to aqueous suspension (198 ng/mL).

Glucocorticoids are widely prescribed in the treatment of ophthalmic inflammations. However, poor aqueous solubility poses a challenge to ophthalmic formulation development. In a recently published report Kassem, et al. have prepared and evaluated nanosuspension formulation of prednisolone, hydrocortisone, and dexamethasone for topical ocular delivery [106]. *In vivo* tissue distribution studies of the glucocorticoids nanosuspensions demonstrated significantly higher levels in anterior chamber tissues relative to solution and microcrystalline suspension of similar compounds. Moreover, investigators also reported a direct relationship between nanosuspension viscosity and ocular bioavailability. Recently, a randomized double-blind clinical study of Sophisen derivatives, 3A Ofteno (1.0% diclofenac sodium w/v), and Modusik-A Ofteno (0.1% CS-A w/v) were performed in 120 healthy volunteers [107]. Topical instillation of 3A Ofteno-diclofenac nanosuspension remained on the ocular surface for longer period with less annoying sensation and irritation. Also, Modusik-A Ofteno-CS-A nanosuspension caused significant improvement in the tear production from baseline 5 to 11 mm.

In a recent patent disclosure (WO 2006/062875) entitled, “Ophthalmic nanoparticulate formulation of a COX-2 selective inhibitor,” investigators have incorporated rofecoxib (COX-2 inhibitor) in the polystyrene nanoparticulate system for ophthalmic applications [108]. Formulation prepared with Poloxamer-407 (0.05% w/w) and HPMC remained physically stable, without any change in particle size up to 4 weeks. Drug levels in anterior chamber ocular tissues, such as the cornea (~6,610 ng·gram/tissue) and aqueous humor (~251
ng·gram/tissue) were significantly higher after topical instillation of nanosuspension. Readers are advised to read the patent for more detailed information of in vivo tissue distribution studies.

The efficiency of nanosuspensions in increasing the drug ocular bioavailability depends upon the intrinsic solubility of drugs in the lachrymal fluid, this intern is governed by the intrinsic dissolution rate of drugs in the lachrymal fluid which can vary due to constant inflow and outflow of lachrymal fluid. Nanosuspensions may therefore fail to give a consistent performance. However, nanosuspension formulation of drugs represents an ideal approach for ocular delivery of poorly soluble drug due to their inherent ability to increase a drug’s saturation solubility [109].

**Nanoemulsion**

The only difference between conventional emulsion and nanoemulsion is the globule size of an internal phase. Nanoemulsion offers several advantages in ocular drug delivery, such as high capacity to dissolve both hydrophilic and lipophilic drugs, stability, improved bioavailability, and good spreadability [16]. In addition, surfactants used in formulating emulsions can also act as penetration enhancers, thereby improving drug permeability across the cornea. Emulsion based delivery of hydrophobic drugs has always remained a primary choice for the formulation researchers.

Chitosan, a cationic polymer, finds application in the field of ocular drug delivery due to its potential ability to enhance corneal drug permeability by opening tight junctions. It strongly interacts with negatively charged mucin and improves residence time on the precorneal surface. An indomethacin-embedded chitosan nanoemulsion was evaluated for its residence time and ability to deliver therapeutics into the anterior chamber of the eye [110].
Topical application of their nanoemulsion has significantly improved indomethacin levels in the cornea and aqueous humor of rabbit eyes relative to the indomethacin solution. Drug levels in the cornea and aqueous humor were about 12-fold higher for the nanoemulsion relative to solution-treated eyes.

A CS-A-loaded microemulsion, in situ electrolyte-triggered gelling system was developed by Gan, et al. [111] for the treatment of corneal allograft rejection. The microemulsion was dispersed in the Kelcogel (deacetylated gellan gum) solution, which provided the in situ gelling property when applied to the corneal surface. In vivo studies suggested that the microemulsion-Kelcogel system can generate ~3-fold higher levels of CS-A relative to CS-A microemulsion even at 32 h post-dosing. Moreover, concentration of CS-A in Kelco gel system treated corneas were maintained at therapeutic levels with no ocular irritation, even after 32 h. In another study, n-octenyl succinate starch was utilized to prepare the diclofenac solution and emulsion, and these formulations exhibit improved permeability across the excised porcine cornea compared to the commercial product Voltaren Ophtha [112]. An indomethacin nanoemulsion prepared with amphoteric surfactant (lauroamphodiacetate) improved corneal permeability by 3.8 times relative to a marketed product (Indocollyre) [113].

In a recently published patent, CS-A was successfully incorporated in a nanoemulsion, utilizing a positively charged polar lipid, such as stearylamine [114]. Mean droplet size of the formulation was within the range of 150-250 nm, with zeta potentials of 34-45 mV. Gan, et al. have recently patented a nanoemulsion-based in situ gelling system for topical ocular delivery of flurbiprofen [115]. A CS-A-loaded nanoemulsion (NOVA22007) containing cationic lipid formulation has just completed Phase III studies for dry eye [116]. The same emulsion was
applied for vernal keratoconjunctivitis treatment and this study has recently completed phase II/III studies [117].

**Nanomicelles**

Polymeric micelles are self-assembling colloidal systems comprised of block or graft amphiphilic copolymers and surface-active agents [118]. Development of ophthalmic drug delivery systems with micelles is very promising, particularly because of their advantages such as high thermodynamic and kinetic stability, ability to sustain the release, and the improvement of drug solubility and permeability across the ocular tissues [119]. Moreover, micelles can be functionalized with endogenous molecules for targeted ocular delivery. Normally, particle size of the micelles ranges from 5 to 50 nm [118].

Recently, a rapamycin and corticosteroid-loaded aqueous nanomicellar formulation was developed and patented (WO2010/144194) by Mitra, *et al.* [120]. Nanomicelles were prepared with 1 to 7% w/v of Vitamin E tocopherol PEG succinate (vitamin E TPGS, HLB-10) and 1 to 3% w/v of octoxynol-40 (HLB-13). Vitamin-E TPGS helped to increase the solubility of the poorly soluble drugs, while octoxynol-40 reduced ocular discomfort and also provided extra stability with higher optical clarity to the nanomicellar formulation. Investigators suggested that any buffer system with adjusted osmolality and physiological pH could be used to prepare an external aqueous phase. Rapamycin has very low aqueous solubility (2.6 µg/mL). However, after preparation of nanomicelles, its solubility improved to 2 mg/mL (~1000 fold). An average diameter of the nanomicelles was around 25 nm. Nanomicellar formulation of $^{14}$C rapamycin was instilled in rabbit eyes, and 60 min post-dosing ocular distribution of the drug was determined by liquid scintillation counter. Noticeably, a higher concentration of $^{14}$C rapamycin was observed in the choroid/retina (~360
ng/g), while very negligible radioactivity was found in the lens, aqueous humor and vitreous humor. Higher accumulations of rapamycin in the posterior segment of the eye was believed to be a result of the smaller mean diameter of nanomicelles. Optically clear and thermodynamically stable nanomicellar formulation could be a promising innovation for the non-invasive treatment of the posterior segment diseases.

In another disclosure (US 2009/0092665), Mitra and co-inventors developed mixed micellar formulation of calcineurin inhibitors (voclosporin) and mTOR inhibitors for ophthalmic applications [121]. Various concentrations of Vitamin-E TPGS and octoxynol-40 were used to prepare nanomicelles. Dilution studies were performed to evaluate the stability of voclosporin-loaded mixed micelles (0.2 wt%), and micellar stability was confirmed up to a 20 fold dilution in saline. In addition, results have demonstrated that micelles dissociated at around 44 °C and the formulation was re-stabilized well within 8 min. Moreover, formulation remained stable in LDPE, polypropylene and polyvinylchloride containers at room temperature for 48 h. The particle size of the voclosporin-loaded (0.2 wt%) nanomicelles was between 13-33 nm. In vivo ocular tissue distribution studies were performed in two groups (single dose and 7 days repeat dose) of rabbits after topical application of mixed micellar formulation-loaded with 14C-radio labeled voclosporin. Almost 1.5-2.5 fold higher C_max were observed in all ocular tissues (cornea, aqueous humor, sclera, upper eyelid, lower eyelid, retina/choroid, lacrimal gland, optic nerve and lower bulbar conjunctiva) of a group with 7 days repeated dosing over to the single dose group. No signs or serious symptoms of irritation were observed in any rabbits after a tolerability and tissue irritability study. According to the results discussed in this disclosure, nanomicellar formulation seems to be very promising in the treatment of anterior and/or posterior segment ocular diseases including AMD and DME.
Results discussed in these patent applications explained that the nanomicellar drug delivery systems can effectively change the course of ocular treatment.

**Cubosomes**

Novel dexamethasone-embedded self-assembled liquid crystalline particles (cubosomes) were developed and investigated for precorneal retention and ocular tissue distribution [122]. The apparent permeability coefficient of dexamethasone, delivered in cubosomes was 3.5–4.5 times higher than dexamethasone eye drops. In addition, precorneal retention of cubosomes was significantly longer than carbopol gel or solution. *In vivo* microdialysis studies were performed to evaluate pharmacokinetics of dexamethasone in aqueous humor. Delivery of cubosomes exhibited 1.8-fold and 8-fold higher AUC 0/240min of dexamethasone in aqueous humor relative to eye drops and suspension, respectively. Moreover, tissue integrity and corneal structure indicated good biocompatibility with the cubosome formulation.

**Ocular implants and inserts**

Intraocular implants have been studied in order to control and sustain the drug delivery in the treatment of proliferative vitreoretinopathy (PVR), cytomegalovirus (CMV) retinitis, glaucoma, endophtalmitis, and posterior capsule opacification. Understanding of physicochemical properties of the active compound and release kinetics helps to design specific ocular implantable drug delivery systems. Ocular implants can be classified in two categories according to their *in vivo* degradability i.e., non-biodegradable implants and biodegradable implants.
Non-biodegradable ocular implants

Since the early in 70s, non-biodegradable implants have offered the advantages of controlled and sustained release of active drugs with minimal host responses. Nowadays, new non-biodegradable polymers (e.g., polyvinyl alcohol (PVA), ethylene vinyl acetate (EVA), and polysulfone capillary fiber (PCF)) have been designed to ensure sustained release for longer period of time with higher biocompatibility [123]. Intraocular drug release kinetics is controlled by the rate of erosion and spontaneous degradation of these polymers. Diffusion of fluid (water) inside the polymer structure dissolves the drug pellet and generates a saturated drug solution inside the implant. Saturated drug solution diffuses out from the implant and provides nearly zero-order drug release [124]. Lack of initial burst release makes non-biodegradable implants superior to implants constructed from biodegradable polymers. Complications such as retinal detachment, endophthalmitis, vitreous hemorrhage, formation of tenacious epiretinal membranes, and cystoids macular edema have been observed with the use of non-biodegradable polymeric devices [125]. Moreover, implantation of this device requires surgical treatment and necessitates surgery to remove the empty device.

Sanborn and colleagues have employed non-biodegradable implants for the ocular delivery of ganciclovir in the treatment of CMV retinitis [126]. These implants were reservoir type devices, composed of drug and coating polymers such as PVA and EVA. PVA is a permeable polymer, which acts as a framework and controls release of ganciclovir. In contrast, EVA acts as an impermeable polymer, which limits the practical surface area of the device and hence, sustains drug release. Implication of both polymers in structure design enables zero-order controlled drug release.
Vitrasert, a commercially available implant of ganciclovir, is relatively large and requires 4-5 mm of sclerotomy at pars plana for implantation [127]. Moreover, the device is made from non-biodegradable polymers and, needs to be removed after 5-8 months for the implantation of another device, or because of unwanted complications such as retinal detachment related to the implant, or severe inflammation. Almost 12% of eye complications (endophthalmitis, retinal detachment, cystoids macular edema, formation of tenacious epiretinal membranes and vitreous hemorrhage) have been observed after implantation of these devices.

To overcome these problems, a betamethasone-loaded intrascleral implant (4 mm in diameter, 1 mm in thickness and 4 mg in weight) composed of PVA and EVA has been developed and evaluated [128]. Results demonstrated that the disc was able to maintain therapeutic levels of betamethasone up to 4 weeks without an adverse burst release of drug. No substantial adverse reactions were observed after histological and electroretinographical evaluations. Intrascleral implantation does not require perforation of the eye wall, hence this system may reduce complications associated with other implanted devices.

The FDA has approved fluocinolone-loaded Retisert in April 2005 in the treatment of chronic non-infectious uveitis [127]. A total of 36 eyes with non-infectious posterior uveitis, which had an average of 2.5 episodes of recurrence annually, were treated with these implants. After implantation of fluocinolone-loaded implants, no recurrence was observed up to two years [129]. Pearson, et al. have prepared Cs-A-loaded PVA-EVA device for the controlled intravitreal delivery of Cs-A [130]. A 500 ng/mL of Cs-A concentrations was observed for more than 6 months in rabbit and cynomolgus monkey eyes. However, complications such as opacity of lens were observed in the implanted rabbit eyes. A PVA-EVA device loaded with
both Cs-A and dexamethasone was prepared, and examined in the treatment of PVR and uveitis [131].

**Biodegradable ocular implants**

Biodegradable polymers generate non-toxic degradation products after *in vivo* enzymatic degradation that can easily eliminate from the body without any side effects. Hence, biodegradable implants are not required to be removed after implantation. It is more difficult to achieve optimal drug release through biodegradable implants when compared to the reservoir type of non-biodegradable implants. The main two categories of biodegradable implants are monolithic and binding types. Monolithic types of implants are made by solidifying the homogenous physical mixture of the drug and polymer. The procedure to produce this kind of implant involves heating of drug, and hence thermolabile drugs such as proteins, polypeptides or nucleic acids are difficult to incorporate in monolithic implants. However, binding type of implants have showed suitability for the delivery of biologically active molecules. Biologically active molecules make chemical bond or a polyion complex with polymers, which finally undergoes enzymatic degradation and gets released from the polymer matrix. Biodegradable polymers can be made into sheets, plugs, rods, pellets and discs, and can be implanted into intrascleral or peribulbar, anterior chamber, vitreous cavity, or through the pars plana [132]. An undesirable final burst release is the major drawback of the controlled release system composed of biodegradable polymers.

Scleral implants of ganciclovir were successfully made using various blends of PLA (DL isomer) and evaluated in the treatment of CMV retinitis in pigmented rabbit eyes. Implants made up from physical mixture of 80% of PLA (70 kDa) and 20% of PLA (5 kDa) were able to maintain therapeutic levels of ganciclovir up to 6 months without any significant burst
Fluorouracil-loaded biodegradable intravitreal implants were prepared from PLGA and evaluated in the treatment of tractional retinal detachment due to PVR [134]. The sustained therapeutic concentration of drug was observed to be above 0.3 μg/mL up to 21 days however, 89% of the rabbit eyes were suffered retinal detachment after treatment. Zhou, et al. have investigated multiple drug delivery through implant in the treatment of PVR [135]. Three cylindrical parts (triamcinolone, human recombinant tissue plasminogen activator (tPA) and 5-fluorouridine) containing PLGA implant (0.8 mm in diameter, 7 mm long) have been prepared and evaluated. This implant exhibited release of 5-fluorouridine and triamcinolone at the maximal rate of 1 μg/day over 4 weeks and 10 - 190 μg/day over 2 weeks, respectively. Moreover, the portion of PLGA coated tPA started release after 2 days at the rate of 0.2 - 0.5 μg/day for 2 weeks, which may minimize the risk of post-operative bleeding. These types of implants unwrap the feasibility of co-delivery of three different drugs or drugs with different release profiles.

Xie, et al. have examined heparin incorporated PLGA implants (HPI) in the treatment of posterior capsular opacification (PCO) [136]. Heparin levels in blood and aqueous humor were investigated after subconjunctival implantation of HPI, posterior chamber implantation of HPI and instillation of 5% topical heparin drops. Implantation of heparin delivery systems in posterior chamber demonstrated significantly higher heparin levels in aqueous humor for longer period of time (12 weeks). A proline analog (cis-4-hydroxyproline (CHP)), inhibits collagen secretion in proliferative vitreoretinopathy (PVR). Yasukawa and colleagues have incorporated CHP in different blends of PLGA (65/35 and 50/50) for the sustained delivery to rabbit eyes [137]. The PLGA 65/35 implants have decreased the incidence of PVR from 89%
to 56% but the PLGA 50/50 implant did not show any significant effect. On the other side, the application of both implants together revealed synergistic inhibition of PVR.

Natural polymers such as gelatin, collagen and alginates have also been studied for the preparation of ocular implants. Koelwel, et al. have prepared and investigated epidermal growth factor (EGF) incorporated alginate inserts in 18 test volunteers in the treatment of keratoconjunctivitis sicca (KCS) [138]. Alginate inserts with higher amount of guluronic acid (G block) were able to sustain the release of EGF for much longer period of time. Dried gelatin hydrogel prepared by cross-linking of acidic gelatin was soaked in basic fibroblast growth factor (bFGF) solution with different concentrations and implanted into rabbit corneal pocket [139]. The inserts with 50 ng or more doses exhibited dose dependent corneal angiogenesis started from day 3 to day 4, while inserts with 20 ng or below were devoid of this side effect.

Moreover, effective sustained delivery of antimetabolic [140, 141], antifungal [142], immunosuppressive agents [143] and steroids [144, 145] have been studied using biodegradable ocular implants. Hence, the employment of implants for the ocular delivery of small molecules (hydrophilic and hydrophobic) and macromolecules is a viable approach.

**Microneedles**

The concept of microneedles was proposed in early 1970s but practically this method not demonstrated until 1990s when the microelectronic industries provided tools to fabricate such small devices. Application of an array of microneedles creates large transport pathways, larger than the molecular dimensions, and thus facilitates the transport of macromolecules (peptides such as insulin, desmopressin and human growth factors), supramolecular complexes, NPs, microparticles and liposomes [146]. Although micro-scale holes in the skin are safer than intraocular injections, safety studies are needed to be performed. Microneedle
based drug delivery is widely studied in transdermal applications, but recently, several studies have been performed to evaluate its application in ocular drug delivery. Mainly, two approaches have been studied for the ocular application of microneedles, solid coated microneedles [147] and hollow microneedles [148]. In the first approach, Jiang, et al. have coated microneedle with five different model drugs (sulforhodamine, sodium fluorescein, bovine serum albumin (BSA), plasmid DNA, and pilocarpine) for intrascleral and intracorneal application to evaluate different model drugs with different physicochemical properties [147]. Microneedles coated with sulforhodamine and BSA demonstrated excellent penetration into human cadaveric sclera and rapid dissolution of coated model drugs. Moreover, intracorneal delivery of sodium fluorescein exhibited higher in vivo levels when compared to the levels achieved after topical application of same dose. The same study using pilocarpine in rabbit eyes has also revealed some promising results.

Moreover, researchers have evaluated the applicability of hollow microneedles for the delivery of solutions containing soluble molecules, NPs, and microparticles into sclera in a minimally invasive manner [148]. The results described successful intrascleral delivery of solutions and fluorescence tagged NPs with insignificant effects of scleral thickness and infusion pressure. However, investigators have reported that the presence of scleral glycosaminoglycans and collagen fibers are the rate limiting factors for the microneedle based intrascleral microparticles delivery.

These studies exhibited the versatility of microneedles’ applications in terms of drug selection (coated microneedles) as well as formulation delivery tool (hollow microneedles). Moreover, these targeted delivery systems are less invasive, less painful and more patient compliant.
Biodegradable polymers [149]

Biodegradable polymers have numerous applications in the field of ocular drug delivery and can be classified as natural or synthetic. Applications of PGA as suture material in the late 1960 provided an impetus for the design and development of novel synthetic biodegradable polymers. Synthetic biodegradable polymers can be tailored in various compositions and molecular weights. The molecular weight or composition regulates the degradation of polymer where the main weight loss takes place due to chain cleavage. Many researchers have investigated several types of biodegradable polymers and studied their applicability for the development sustained ocular formulations. Structures of various biodegradable polymers are depicted in Figure 1.4.

Polyalkylcyanoacrylates (PACA)

Polyalkylcyanoacrylates (PACA) belong to a class of acrylate polymers synthesized from alkylcyanoacrylic monomers through anionic polymerization. The faster degradation rate of PACA was attributed to the unique instability of a carbon-carbon sigma bond and presence of electron withdrawing neighboring groups. It has shown remarkable applicability as surgical glue and skin adhesive. PACA was also explored for the development of NPs. The PACA degradation rate varies from hours to days depends on the length of alkyl side chain. For example, PACA having shorter alkyl chain length such as polymethylcyanoacrylate degrades in few hours whereas higher alkyl chain length derivatives such as octyl and isobutyl cyanoacrylates degrade slowly. NPs composed of PACA are advantageous in terms of fabrication and application in drug delivery [150].
**Polyanhydrides**

Numerous investigations have elucidated the role of polyanhydrides (POA) for ocular drug delivery applications. These polymers exhibit faster degradation and limited mechanical strength, which make them an ideal candidate for fabrication of sustained release devices. Low molecular weight polyanhydrides were synthesized through dehydrative coupling and dehydrochlorination whereas melt polycondensation polymerization was employed for synthesis of high molecular weight polymers. The degradation rate of polyanhydrides can be easily modulated by changing the polymer composition and depends on the crystallinity and hydrophilicity of the final polymer. These polymers undergo surface erosion and generate monomeric acids that are non-toxic. Homo-polyanhydrides have limited application in controlled drug delivery due to their crystalline nature. In contrast, copolymers such as poly(carboxyphenoxy)propane–sebacic acid (PCPP-SA) demonstrated controlled degradation rates [151]. This polymer was approved by the FDA for human applications for the delivery of carmustine in the treatment of brain cancer [152]. Other approaches based on aromatic co-monomers composed of hydrophobic aliphatic linear fatty acids were also investigated for drug delivery applications [153].

**Polyesters**

Polyesters are biodegradable polymers having short aliphatic, ester-linked backbones. These classes of polymers are generally produced by either ring-opening or condensation polymerization. Ring-opening polymerization is preferred over condensation reaction to produce high molecular weight polyesters. Homo- or co-polymers of cyclic lactones and anhydrides having narrow molecular weight distribution can be produced via ring-opening polymerization. The molecular weight of the final polymer can be controlled by varying the
ratio of monomers. The molecular weight of the polyesters regulates the hydrolytic cleavage that follows bulk erosion kinetics to produce metabolic products which are eliminated through normal metabolic pathways [154]. The hydrolytic degradation rate of the polymers can be altered by varying the molecular weight, crystallinity and structure of the polymeric chain. Among polyesters, poly-α-hydroxyesters are the most broadly investigated class of polymers for ocular drug delivery applications; this includes PGA, PLA and their copolymers.

**Polycaprolactone (PCL)**

PCL is a semicrystalline polymer synthesized by ring-opening polymerization of ε-caprolactone. It has a glass transition temperature of -60 °C and a melting temperature in the range of 59 to 64 °C. PCL was investigated for long term delivery due to higher permeability to many drugs, excellent biocompatibility and extremely slow hydrolytic cleavage of polyester backbone. The PCL based Capronor® implant was developed for controlled delivery of levonorgestrel. It has a low tensile strength of 23 MPa and an extremely high elongation factors, more than 700%. Numerous investigations were attempted to improve the slower degradation of PCL. Copolymers of ε-caprolactone with lactide or glycolide exhibits remarkably better degradation profile.

**Polyglycolide (PGA)**

PGA is a relatively hydrophilic polymer compared to other polyesters with high crystallinity and low solubility in organic solvents. The low solubility in organic solvent is attributed to its higher tensile modulus. It has a high melting point of 225 °C and glass transition temperature of 36 °C. It has a comparatively faster degradation rate than other polyesters and generates glycine upon degradation, which eventually eliminates through the citric acid cycle. Major losses in the mechanical strength of PGA usually take place in one to two months and
it completely degrades \textit{in vivo} within six to twelve months. PGA was initially explored for developing sutures because of their fiber-forming properties and excellent mechanical strength. However, it has limited role in ocular drug delivery due to its faster degradation rate and higher crystallinity. PGA implants can be easily fabricated by widely applicable processing techniques such as solvent casting, compression and extrusion techniques. Processing technique utilized for the production of implant regulates the degradation properties of implant [155].

\textbf{Polylactic acid (PLA)}

PLA is comparatively more hydrophobic than PGA due to the presence of an additional methyl group. It is chiral in nature because of the structure of lactic acid and commonly exists in three isomeric forms the D (-), L (+) and racemic (D, L) lactide. The crystalline nature of PLA depends upon the isomeric forms and molecular weight of the polymer. PLA (L) is crystalline in nature and hydrolyzed through normal metabolic pathway due to presence of naturally occurring isomer (L-lactide). It has a melting point of 175 °C and glass transition temperature of 60 to 65 °C. It also possesses a good tensile strength of 50-70 MPa and high modulus of 4.8 GPa. On the other hand, PLA (DL) is amorphous in nature due to presence of the racemic mixture. It has glass transition temperature of 55 to 60 °C and comparatively faster degradation rate than PLLA. All isomeric forms of PLA follow bulk erosion kinetics and generate lactic acid upon hydrolytic cleavage [116]. Researchers have often utilized PLA for ocular drug delivery applications.

\textbf{Poly (lactide-co-glycolide) (PLGA)}

Poly (lactide-co-glycolide), commonly known as PLGA is obtained by the copolymerization of lactide and glycolide. This copolymer is hydrolytically less stable than
the homopolymers, PLA or PGA. Extensive research on a full range of these copolymers suggests their implication in drug delivery. These copolymers are divided into two main compositions, comprised of lactide and glycolide. PLGA follows bulk erosion kinetics, and its degradation depends on molecular weight and lactide to glycolide ratio. As the ratio of glycolide in copolymer decreases, the hydrolytic degradation rate decreases. The intermediate PLGA, i.e. 50:50, hydrolyses faster than PLGA 75:25 and PLGA 85:15. PLGA copolymers are FDA approved for human applications because of the excellent biocompatibility and controlled degradation profiles [117]. PLGA is negatively charged and thus has a non-mucoadhesive nature.

**Polyorthoesters**

Polyorthoesters (POE) are hydrophobic polymers composed of a hydrolytically unstable polyester linkage. However, they exhibit slower degradation due to surface erosion. This degradation characteristic is ideal for designing sustained release devices. In this polymer the degradation profile can be easily adjusted by employing different diols for polymerization [156]. The POE group is hydrolytically unstable in acidic conditions and requires basic additives to inhibit autocatalysis. The first generation of POE was developed by the ALZA Corporation. It was synthesized by the transesterification reaction of diol with diethoxy tetrahydrofuran [157]. Degradation profile of POE II can be easily altered by incorporation of acidic additives such as adipic acid. POE III upon hydrolysis generates diol and pentaerythriol dipropionate, which subsequently generate propionic acid and pentaerythriol. This class of polymers is biocompatible and follows pH dependent degradation behavior. They do not require organic solvents for the incorporation of drugs due to their semisolid nature. However, there are difficulties in scale up processes that limit their application in drug delivery.
Modification of second generation polyester (POE II) with smaller lactic or glycolic acid chains led to the development of POE IV. This polymer upon hydrolysis liberates acids which further promotes the polymer degradation. The physical form and degradation rate of the polymer can be easily varied by changing diols and acid segment respectively. POE IV has demonstrated good biocompatibility for controlled delivery applications [158].
Figure 1.4: Structure of biodegradable polymers
CHAPTER 2

INTRODUCTION

Statement of the problem

The retinal pigment epithelium (RPE), macular region, choriocapillaries and Bruch’s membrane are the primary target sites of vision threatening diseases such as dry and wet AMD, proliferative vitreoretinopathy, DR and DME [159]. Out of all these diseases, wet AMD is believed to be a major cause for severe central vision loss or legal blindness in the individuals 65 years or older [160]. Elevated levels of vascular endothelial growth factor (VEGF) are reported in choroidal neovascularization (CNV), and it is a primary reason for the development of wet AMD. Occurrence of CNV is followed by leakage of fluid and blood into the subretinal space and subsequent scar formation, which eventually leads to irreversible vision loss [161].

Therefore, anti-VEGF antibodies such as bevacizumab and ranibizumab are recommended in the treatment of wet AMD [162]. Bevacizumab (149 kDa) is a full-length recombinant humanized murine monoclonal antibody (rhum-anti-VEGF antibody) specific to all isoforms of VEGF [163]. It specifically binds to the extracellular VEGF and blocks the angiogenic action of VEGF. At a dose of 1 mg or 1.25 mg [164], it has shown reduction in the macular thickness, reduction in angiogenic leakage, and improvement in visual acuity of neovascular AMD patients [165, 166]. Ranibizumab is a 48 kDa Fab-fragment of a humanized murine anti-VEGF antibody active against all isomers of VEGF. It is one-third the size of bevacizumab and use the same molecular mechanism to block the VEGF [167]. Due to short half-life, current anti-VEGF therapy with bevacizumab or ranibizumab requires frequent intravitreal injection to maintain therapeutic levels at retina/choroid. However, treatment with frequent intravitreal
administrations is associated with many potential complications like endophthalmitis, retinal detachment, retinal hemorrhage, and patient non-compliance [168].

Therefore, there is a need to develop a novel formulation for the sustained delivery of macromolecules (e.g., antibodies). Sustained delivery systems such as NPs may offer an advantage of higher residence time for macromolecules at the site of absorption. Drug encapsulation within NPs (1-1000 nm) can be achieved with most commonly used biodegradable and biocompatible polymers such as PLA, PCL, and PLGA. However, in vivo degradation of PLA and PLGA NPs produces high molar masses of lactic acid and glycolic acid, which reduces the pH in microenvironment. This pH change leads to the higher hydrolytic degradation of protein therapeutics, and also causes tissue irritation and toxicity [169]. In addition, rapid degradation of PGA or PLGA based formulation results in significantly higher burst release followed by faster rate of release [170, 171]. On the other hand, the rate of degradation for PLA or PCL based copolymers are very slow. Poor degradation of PCL based formulation leads to accumulation of formulation (without drug) in limited vitreous space, which is highly unacceptable.

**Hypothesis**

There is a need to develop novel polymeric system which can sustain the release of macromolecules and also improves the stability of the same. Therefore, preparation of novel biodegradable PB copolymers, and their applications for the development of macromolecules-loaded NPs and thermosensitive gel have been proposed in this research protocol. Various macromolecules such as catalase (237 kDa), IgG (150 kDa), IgG-Fab (48 kDa), bovine serum albumin (BSA) (66 kDa), lysozyme (14.7 kDa), insulin (5.8 kDa), octreotide (1 kDa), and bevacizumab (149 kDa) loaded formulations have been investigated in this research work.
The PB copolymers are comprised of FDA approved polymer blocks such as PEG, PCL and a small molar mass of PLA/PGA. Tailoring of the molecular weight or interplay of the sequence of blocks can provide different hydrophilic or hydrophobic polymers. The presence of PEG in the PB copolymers will allow improving the physical stability of NP formulation. In addition, it will also help to enhance the stability of peptide/protein molecules by avoiding the direct interaction between hydrophobic segments of polymers with peptide/protein. PEG will also decrease the direct contact of protein therapeutics with the organic solvents during the NP preparation. PCL is the second important block of PB copolymers. In corporation of highly crystalline PCL block in the backbone of PB copolymers will significantly improve DL and drug EE of PB NPs. Furthermore, slower degradation of PCL blocks will sustain the drug release for significant period of time. PB copolymers are also composed of small molar mass of PLA/PGA. Incorporation of PLA/PGA in PB copolymers significantly reduces crystallinity of PCL which eventually accelerates hydrolytic degradation of PCL. Alteration of polymer degradation rate will shift the drug release kinetics from diffusion controlled to degradation controlled. Hence, by these structural modifications in PB copolymers, nearly zero-order drug release can be easily achieved. The second important reason behind designing of PB copolymer is to minimize the amount of lactic acid produced upon in vivo degradation. It is anticipated that reduction of the PLA molar mass in PB copolymers will improve the stability of encapsulated protein molecules, and it will also reduce possible tissue irritation and toxicity.

This novel approach will provide the entire range of polymers with different hydrophilicity-hydrophobicity index. This enormous advantage will allow us to select a unique composition of polymer which will be best suited for a respective therapeutic agent. Furthermore, reduction in the molecular weight and/or change in the polymer block
arrangement will allow us to prepare PB copolymer with totally different and unique physicochemical property i.e., temperature sensitivity. These new compositions will be water soluble at room temperature but upon increase in temperature, they will transform to solid hydrogel.

Catalase (237 kDa), IgG (150 kDa), IgG-Fab (48 kDa), BSA (66 kDa), lysozyme (14.7 kDa), insulin (5.8 kDa), and octreotide (1 kDa) will be used as model proteins for the optimization of formulation components and processes. PB copolymers will be utilized for the preparation of NPs and thermosensitive gel. Many investigators have reported significant burst release of therapeutic agents (peptides/proteins) when delivered from NPs or hydrogel systems [170, 171]. This problem may be eliminated by introducing dual approach or composite formulations, in which drug-loaded NPs are suspended in thermosensitive gel. This composite approach may minimize the burst release effect and provide nearly zero-order drug release by offering longer drug diffusion pathway across the system.

Subconjunctival/intravitreal administration of such a novel formulation may result in a prolong duration of action (approximately 6 months) and thereby eliminate the need for repeated administration. It is anticipated that this formulation will provide higher patient compliance with reduced side effects. This approach may act as a platform for ocular delivery of other therapeutic macromolecules such as siRNA, aptamers, peptides and large proteins. Therefore, the broad objective of this research is to develop novel PB copolymer based sustained delivery systems of various peptide/protein therapeutics for the treatment of posterior segment diseases.
Objectives

Objectives of this research are as follow,

1) To synthesize and characterize various triblock (TB) and PB copolymers for the preparation of thermosensitive hydrogels. In addition, the effects of various parameters including molecular weight, arrangement of blocks and hydrophobicity on sol-gel transition, viscosity and in vitro drug release will be investigated. The possible mechanism of sol-gel transition will also be evaluated.

2) To synthesize and characterize various TB and PB copolymers for the preparation of IgG-loaded PB NPs. The effect of molecular weight, hydrophobicity and isomerism on EE, loading and in vitro release of IgG will be investigated.

3) To synthesize and characterize various PB copolymers for the preparation of protein-encapsulated NPs. More importantly, the effect of hydrophobicity of PB copolymer on the encapsulation of different proteins (IgG, BSA and Bevacizumab) will be investigated. The effect of size and hydrodynamic diameter of protein on various formulation parameters will also be studied. The stability of released protein will be investigated by chemical and biological assays. Approaches to composite formulations (NP suspended in thermosensitive gel) to achieve zero order drug release will be investigated.

4) In order to achieve maximum DL and zero-order drug release, various formulation process parameters including addition of NaCl will be optimized.

5) In vitro and in vivo polymer tolerability studies will be performed.
CHAPTER 3
NOVEL THERMOSENSITIVE PENTABLOCK (PB) COPOLYMERS FOR
SUSTAINED DELIVERY OF PROTEINS IN THE TREATMENT OF POSTERIOR
SEGMENT DISEASES

Rationale

Bevacizumab, a chimeric anti-VEGF antibody (149 kDa) approved for treatment of colon cancer is being used off-label for the 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 [165, 166]. However, due to the chronic nature of ocular diseases and shorter intravitreal half-life of this anti-VEGF antibody, frequent intravitreal injections are indicated to maintain therapeutic activity in the retina and choroid. Frequent administrations are inconvenient and cause potential complications such as endophthalmitis, retinal detachment, retinal hemorrhage, and more importantly, patient non-compliance [172-174]. Since the wide application of protein therapeutics in many treatments including ophthalmology, tumor biology and immunology, pharmaceutical scientists are more focused towards the development of novel delivery strategies utilizing current therapeutics rather than on development of new drugs. Development of sustained release formulations of protein therapeutics can reduce the frequency of intravitreal injections and eventually eliminate potential complications. This approach may lower 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 [175]. 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 as soon as it is exposed to body temperature, it immediately phase transforms into a solid hydrogel polymer matrix. The hydrogel matrix protects protein therapeutics from enzymatic degradation and provides sustained release for a longer period of time, eliminating repeated monthly injections. Various biodegradable copolymers composed of hydrophobic polymer blocks including PCL, PLA, polyglycolide (PGA) and PLGA, and hydrophilic polymer blocks, in particular PEG, have been investigated for their thermosensitive behavior [176-178]. Adjustment of the hydrophilic-hydrophobic balance in the block copolymer backbone allows manipulation of the sol-gel transition curve.

Many researchers have synthesized and 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 formulations [176, 179]. However, applicability of PCL based TB copolymers (A-B-A or B-A-B) as thermosensitive gels is not very suitable due to their poor biodegradability and faster drug release [180]. Slow degradation of PCL is attributed to its highly crystalline nature [181]. Degradation of PLGA/PLA/PGA produces lactic acid and glycolic acid, and significantly reduces pH in the microenvironment and hence triggers the degradation of protein therapeutics [182]. Moreover, PLGA also induces structural changes in protein molecules by the process of acylation [182, 183]. Therefore, an alternative development of novel polymeric system which reduces the molar mass of lactic acid/glycolic acid upon degradation becomes pivotal. It is also crucial to establish coordination between degradation profile and drug release profile. To date, very limited studies utilizing
thermosensitive hydrogels have been established for the treatment of posterior segment neovascular diseases.

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

**Materials and methods**

**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). Hexamethylene diisocyanate (HMDI) and Micro-BCA™ were obtained from Fisher scientific. Mouse TNF-α, IL-6 and IL-1β (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) and PB copolymers with C-B-A-B-C (PLA-PCL-PEG-PCL-PLA) block arrangements

The PCL-PEG-PCL TB copolymers were synthesized by ring-opening bulk copolymerization of ε-caprolactone [176]. PEG was utilized as macroinitiator and stannous octoate as a catalyst. Briefly, before polymerization, PEG (1, 1.5, 2 and 4 kDa) were vacuum dried for 4 h. A predetermined amount of PEG (4.0 g) and ε-caprolactone (8.0 g) were added in the round bottom flask. The polymer melt was degassed under vacuum for 30 min at 130 °C. The flask was then purged with nitrogen gas, followed by addition of stannous octoate (0.5 wt%). The 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. Precipitate was centrifuged and vacuum-dried to remove residual solvents. Purified polymers were stored at -20 °C. A schematic synthesis scheme is presented in Figure 3.1a.

Synthesis of PB copolymers 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 PB copolymers A (PB-1), B (PB-2) and C (PB-3) (Table 3.1). To synthesize PB copolymers, 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. The flask was then purged with nitrogen gas and followed by addition of stannous octoate (0.5 wt%). The reaction was carried out at 130 °C for 24 h. The resulting polymers were purified and stored in a similar manner as described earlier.
**Step 1**

\[
\text{PEG} + \text{g caprolactone} \xrightarrow{\text{Sn(Oc)}_2, 130^\circ C} \text{HO-PCL-PEG-PCL-OH}
\]

**Step 2**

\[
\text{HO-PCL-PEG-PCL-OH} + \text{Lactide} \xrightarrow{\text{Sn(Oc)}_2, 130^\circ C} \text{PLA-PCL-PEG-PCL-PLA}
\]

**Figure 3.1a:** Synthesis scheme for the TB-1, TB-2, TB-3, TB-4, TB-5, PB-1 and PB-2 copolymers
Figure 3.1b: Synthesis scheme for the PB-4 and PB-5 copolymers
### Table 3.1: List of TB and PB copolymers studied

<table>
<thead>
<tr>
<th>Code</th>
<th>Structure</th>
<th>PLA/PCL/PEG</th>
<th>Total $M_n^a$ (theoretical)</th>
<th>Total $M_n^b$ (calculated)</th>
<th>Total $M_n^c$ (calculated)</th>
<th>$M_n^c$ (GPC)</th>
<th>PDI$^c$</th>
<th>Solubility in water</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB-1</td>
<td>PCL$<em>{1000}$-PEG$</em>{1000}$-PCL$_{1000}$</td>
<td>0/2/1</td>
<td>3000</td>
<td>3050</td>
<td>3430</td>
<td>4720</td>
<td>1.37</td>
<td>Soluble</td>
</tr>
<tr>
<td>TB-2</td>
<td>PCL$<em>{1500}$-PEG$</em>{1500}$-PCL$_{1500}$</td>
<td>0/2/1</td>
<td>4500</td>
<td>4580</td>
<td>4900</td>
<td>6760</td>
<td>1.38</td>
<td>Soluble</td>
</tr>
<tr>
<td>PB-1</td>
<td>PLA$<em>{250}$-PCL$</em>{1250}$-PEG$<em>{1500}$-PCL$</em>{1250}$-PLA$_{250}$</td>
<td>0.33/1.67/1</td>
<td>4500</td>
<td>4520</td>
<td>4750</td>
<td>6640</td>
<td>1.40</td>
<td>Soluble</td>
</tr>
<tr>
<td>PB-2</td>
<td>PLA$<em>{500}$-PCL$</em>{1250}$-PEG$<em>{1500}$-PCL$</em>{1250}$-PLA$_{500}$</td>
<td>0.67/1.67/1</td>
<td>5000</td>
<td>4980</td>
<td>5250</td>
<td>6900</td>
<td>1.32</td>
<td>Soluble</td>
</tr>
<tr>
<td>PB-4</td>
<td>PEG$<em>{550}$-PCL$</em>{550}$-PLA$<em>{1100}$-PCL$</em>{550}$-PEG$_{550}$</td>
<td>1/1/1</td>
<td>3300</td>
<td>3220</td>
<td>4270</td>
<td>6290</td>
<td>1.47</td>
<td>Soluble</td>
</tr>
<tr>
<td>PB-5</td>
<td>PEG$<em>{550}$-PCL$</em>{825}$-PLA$<em>{550}$-PCL$</em>{825}$-PEG$_{550}$</td>
<td>0.5/1.5/1</td>
<td>3300</td>
<td>3270</td>
<td>4330</td>
<td>6100</td>
<td>1.41</td>
<td>Soluble</td>
</tr>
<tr>
<td>TB-3</td>
<td>PCL$<em>{2000}$-PEG$</em>{2000}$-PCL$_{2000}$</td>
<td>0/2/1</td>
<td>6000</td>
<td>5870</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Insoluble</td>
</tr>
<tr>
<td>TB-4</td>
<td>PCL$<em>{4000}$-PEG$</em>{4000}$-PCL$_{4000}$</td>
<td>0/2/1</td>
<td>12000</td>
<td>11850</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Insoluble</td>
</tr>
<tr>
<td>PB-3</td>
<td>PLA$<em>{750}$-PCL$</em>{1250}$-PEG$<em>{1500}$-PCL$</em>{1250}$-PLA$_{750}$</td>
<td>1/1.67/1</td>
<td>5500</td>
<td>5330</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Insoluble</td>
</tr>
</tbody>
</table>

a: Theoretical value, calculated according to the feed ratio.
b: Calculated from $^1$H-NMR.
c: Determined by GPC analysis.
Synthesis of PB copolymers with A-B-C-B-A (PEG-PCL-PLA-PCL-PEG) block arrangements

PB copolymers, PB-4 and PB-5 were synthesized by ring-opening copolymerization where mPEG (550) was utilized as macroinitiator and stannous octoate (0.5 wt%) as a catalyst [176]. Firstly, TB copolymers, mPEG-PCL-PLA were synthesized by ring-opening copolymerization in a manner described earlier. The resulting TB copolymers were coupled utilizing hexamethylene diisocyanate (HMDI) as a linker to prepare PEG-PCL-PLA-PCL-PEG PB copolymers. 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 Figure 3.1b.

Characterization of PB copolymers

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 estimated utilizing a Varian 400-MHz NMR spectrometer. NMR spectra 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 a Styrage 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 PB copolymers were analyzed at room temperature by MiniFlex automated X-ray diffractometer (Rigaku, The Woodlands, Texas, USA) equipped with Ni-filtered Cu-ka radiation (30 kV and 15 mA). The diffraction angle ranged from 5 to 45° using a 1° per min 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 [184]. Briefly, block copolymers ranging from 15-30 wt% were dissolved in distilled deionized water followed by 12 h 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 water bath was raised gradually from 10 to 60 °C at an increment of 1 °C. Vials were kept for 5 min at each temperature. The 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 solution transforms to gel phase was considered as critical gelling temperature (CGT) and the temperature where a polymer starts to precipitate (phase separation) was described as critical precipitation temperature (CPT).
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 medium (DMEM)/F-12 containing 10% heat-inactivated fetal bovine serum (FBS), sodium bicarbonate (2 mM), HEPES (15 mM), 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 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 x 10⁴ per well. 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. The 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,

\[
LDH \text{ release}(\%) = \frac{\text{Abs. of Sample} - \text{Abs. of negative control}}{\text{Abs. of positive control} - \text{Abs. of negative control}} \times 100 \quad \text{...Eq. 3.1}
\]
MTS assay

MTS assay was performed according to a previously published protocol with minor modifications [185]. Briefly, ARPE-19 and RAW-264.7 cells at a density of 1.0 x 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, the 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 100μL 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% cell viability were considered as non-toxic. Percent cell viability was estimated by following equation.

\[
\text{Cell viability (\%)} = \frac{\text{Abs. of Sample} - \text{Abs. of negative control}}{\text{Abs. of positive control} - \text{Abs. of negative control}} \times 100 \quad \ldots \text{Eq. 3.2}
\]

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 [185]. Briefly, 5.0 x 10^4 cells were seeded per well of 48-well plates and incubated for 24 h. After incubation, the 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, the 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 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 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, a similar experiment was 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 in various kinetic models including the Korsmeyer-Peppas, Higuchi, Hixon-Crowell, first-order and zero-order.

**Korsmeyer-Peppas equation**

$$\frac{M_t}{M_\infty} = kt^n$$  \hspace{1cm} \text{...Eq. 3.3}

k is the kinetic constant and n is the diffusion exponent describes release mechanism. $M_t$ and $M_\infty$ represent the cumulative IgG release at time t and at the equilibrium, respectively.

**Higuchi equation**

$$Q_t = Kt^{1/2}$$  \hspace{1cm} \text{...Eq. 3.4}
K denotes the Higuchi rate kinetic constant, \( Q_t \) is the amount of released IgG at time \( t \), and \( t \) is time in hours.

**Hixon-Crowell equation**

\[
C_0^{1/3} - C_t^{1/3} = kt
\]

...Eq. 3.5

\( C_0 \) and \( C_t \) represents the initial amount and remaining amount of IgG in gel, respectively. \( k \) is the constant incorporating surface-volume relation and \( t \) is time in hours.

**First order equation**

\[
\log C = \log C_0 - \frac{Kt}{2303}
\]

...Eq. 3.6

\( K \) denotes the first order rate constant, \( C_0 \) is the initial IgG concentration and \( t \) represents time in hours.

**Zero order equation**

\[
C = K_0 t
\]

...Eq. 3.7

\( K_0 \) is the zero-order rate constant and \( t \) is time in hours.

**\(^1\)H-NMR for coumarin-6-loaded gel**

Five mg of PB-1 copolymer was dissolved in either CDCl\(_3\) or D\(_2\)O followed by addition of 0.5 mg of coumarin-6 a (hydrophobic dye). Samples containing both polymer and hydrophobic dye were subjected to \(^1\)H-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 a particle size analyzer (ZetasizerNano 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 15 wt% 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 ± standard deviation).

**Results and discussion**

**Synthesis and characterization of block copolymers**

FTIR spectrum of PB-4 is reported in Figure 3.2. Absorption band at 1725 cm⁻¹ and multiple bands ranging 1000-1300 cm⁻¹ established the presence of ester linkages in PB copolymer. C-H stretching bands at 2936 and 2865 cm⁻¹ depicted presence of PCL blocks. Absorption band at 1534 cm⁻¹ (N-H banding) and 3344 cm⁻¹ (N-H stretching) exhibited the formation of urethane group in PB-4 copolymer.

¹H-NMR was employed to characterize PCL-PEG-PCL, PLA-PCL-PEG-PCL-PLA and PEG-PCL-PLA-PCL-PEG copolymers. Figure 3.3 depicts the ¹H-NMR spectra of TB-1, PB-1 and PB-4 block copolymers in deuterated chloroform. As described in Figures 3.3a, 3.2b and 3.2c, characteristic ¹H-NMR peaks were observed at 1.40, 1.65, 2.30 and 4.06 ppm.
corresponding to the methylene protons of \(-(\text{CH}_2)_3\)-, \(-\text{OCO-CH}_2\)-, and \(-\text{CH}_2\text{OOC-}\) of PCL units, respectively. A sharp peak at 3.65 ppm was attributed to the methylene protons \(-\text{CH}_2\text{CH}_2\text{O-}\) of PEG. Typical signals (Figures 3.3b and 3.3c) at 1.50 \(-\text{CH}_3\) and 5.17 \(-\text{CH}-\) ppm were assigned for PLA blocks. Whereas, a peak (Figure 3.3c) at 3.38 ppm was denoted to terminal methyl of \(-\text{OCH}_3\)- of PEG.

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

The molecular weight (Mw and Mn) and polydispersity of polymers were determined by GPC. Typical GPC curves of TB-1, PB-1 and PB-4 are shown in Figure 3.4. A single peak for each polymer was observed describing unimodel 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 a narrow distribution of molecular weights. \(^1\text{H-NMR}\) and GPC were applied to calculate molecular weight of block copolymers (Table 3.1). As summarized in Table 3.1, experimental values were consistent with theoretical values derived from feed ratios. 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 (Figure 3.5). Two sharp peaks were observed at \(\theta = 21.5^\circ\) and \(23.9^\circ\) which belong to PCL blocks. No peaks for PEG or PLA were observed. Interestingly, only TB-1, TB-2, PB-1 and PB-2 exhibited crystalline peaks of PCL, 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 a semi-crystalline structure even after covalent conjugation with PEG blocks.
Interestingly, conjugation of PLA blocks at the terminals of TB copolymers exhibited significant reduction in 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 enhanced degradation of block polymer [181]. Hence, it is anticipated that PB-4 and PB-5 might cause faster rate of degradation relative to TB-1, TB-2, PB-1 and PB-2 copolymers.
Figure 3.2: FTIR spectrum of PB-4 (PEG_{550}-PCL_{550}-PLA_{1100}-PCL_{550}-PEG_{550}).
Figure 3.3: $^1$H 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 3.4: 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 3.5: XRD patterns of block copolymers.
**Sol-gel transition**

The block copolymers reported in this study are aphophilic in nature containing hydrophilic block (PEG) and hydrophobic block(s) (PCL and/or PLA). In the case of TB copolymers (TB-1 and TB-2), increased total molecular weight, but 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. This may be attributed to longer PCL chains, which may significantly enhance intermolecular and intramolecular hydrophobic interactions of polymer and outweigh the ability of PEG to solubilize polymer. PB-1 and PB-2 copolymers are easily soluble in water and exhibit sol-gel transition behavior. However, the increase in hydrophobicity by increasing molecular weight of PLA chain (PB-3) exhibited poor aqueous solubility, which might be due to limitation of PEG to solubilize polymer. For all block copolymers, an increase 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 the block copolymers, sol-gel transition curves of TB-1 and TB-2 were compared (Figure 3.6). With an increase in total molecular weight of block copolymers from 3000 (TB-1) to 4500 (TB-2), CGT decreased whereas the CPT increased to higher values. The temperature range for the 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

Hydrophobicity of block copolymers can be increased by enhancing the molecular weight of the whole 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 a greater hydrophobicity of PB-2 copolymer. It is important to note that a PLA block is less hydrophobic relative to a 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 of PB-1 and PB-2 (Figure 3.7a), PB-4 and PB-5 (Figure 3.7b), and TB-2 and PB-1 (Figure 3.7c) were compared to understand the effect of hydrophobicity on the sol-gel behavior of block copolymers. As described in Figure 3.7a, increased hydrophobicity of PB-2 has significantly reduced the CGT and shifted the value of CPT to higher temperature. A similar behavior was observed when the sol-gel transition curves of PB-4/PB-5, and TB-2/PB-1 were compared.

Higher hydrophobicity polymers may enhance intramolecular and intermolecular hydrophobic interactions even at lower temperatures compared to hydrophilic copolymers which lead to lower CGT. Additionally, these hydrophobic interactions allow for a 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 (C-B-A-B-C) and PB-5 (A-B-C-B-A) were compared (Figure 3.8). 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.
Figure 3.6: Sol-gel transition curves of TB-1 and TB-2.
Figure 3.7: Sol-gel transition curves (a) PB-1/PB-2, (b) PB-4/PB-5, and (c) TB-2/PB-1.
Effects of total molecular weight, arrangement of blocks and hydrophobicity of PB copolymers on sol-gel transition behavior are in agreement with previously published reports of PLGA-PEG-PLGA [186] and PEG-PLGA-PEG [187] copolymer hydrogels.

**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 (Figures 3.9a and 3.9b) 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 the 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 Figures 3.10a and 3.9b demonstrate that more than 90% of cells are viable 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.
Figure 3.8: Sol-gel transition curves of PB-1 and PB-5.
Figure 3.9: *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 3.10: 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 3.11: 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.
**In vitro biocompatibility study**

Estimation of inflammatory mediators such as TNF-α, IL-6 and IL-1β secreted in cell supernatant is a good measure to confirm biocompatibility of block copolymers. These cytokines were estimated in the supernatant of RAW-264.7 cells after 24 h of polymer exposure. Levels of cytokine were estimated by sandwich ELISA method according to manufacturer’s protocol. Our results (Figures 3.11a, 3.11b, and 3.11c) indicate that at 20 mg/mL polymer concentration, release of cytokines was negligible with no significant difference to negative control. Negligible release of TNF-α, IL-6 and IL-1β suggest excellent biocompatibility of PB copolymers with low toxicity.

**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 Figure 3.12. Release of IgG was noticeably affected by the chemical composition of block copolymers. TB copolymers (TB-1 and TB-2) exhibited significantly higher burst release (~40-45% of initial dose) relative to PB copolymers (PB-1, PB-2, PB-4 and PB-5) which showed burst release between ~24-31% of initial dose. Moreover, release of IgG from PB copolymer 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, Figure 3.5) and hydrophobicity of copolymers. This
reduction in hydrophobicity of PB copolymers may have increased the affinity with IgG resulting in prolonged release of IgG from PB copolymers relative to TB copolymers.

In addition, the effect of polymer concentration on the rate of IgG release was also studied. As depicted in Figure 3.13, 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 retards drug release rate, indicating direct relationship of polymer concentration on drug release. At higher polymer concentration, the gels may form compact structures with a smaller porosity in the gel matrix relative to lower polymer concentration. This structural change may lower the diffusion of IgG across gel matrix resulting in lower initial burst release and prolonged 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 kinetics of IgG release (Table 3.2). The Korsmeyer-Peppas model was found to be the best fit model based on \( R^2 \) value. The diffusion exponent, \( n \), ranged from 0.272 - 0.386 for all gelling polymers. The \( n \)-values below 0.43 indicates a diffusion controlled mechanism of IgG release.

**\(^{1}H\)-NMR of Coumarin-6-loaded polymer solutions**

In order to understand the process of polymer solubilization and behavior of sol-gel transition, \(^{1}H\)-NMR of PB-1 copolymer was carried out in CDCl\(_3\) (Figure 3.14a) and D\(_2\)O (Figure 3.14b) spiked with coumarin-6. Coumarin-6 is insoluble in water however aqueous solubility is 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 CDCl$_3$ exhibited sharp peaks representing various protons of PEG, PCL, PLA and coumarin-6. Interestingly, a sample containing PB-1 copolymer and coumarin-6 in D$_2$O was devoid of signals of coumarin-6 suggesting that coumarin-6 is inside the miceller core. Broad peaks observed at 3.65 ppm and 2.30 ppm were attributed to the protons of PEG (-CH$_2$-CH$_2$-) and PCL (-OCO-CH$_2$-), respectively. Proton peaks for PLA and coumarin-6 were absent. Exactly, similar behavior was also observed with the sample containing PB-5 copolymer and coumarin-6 simultaneously dissolved in CDCl$_3$ (Figure 3.14c) or D$_2$O (Figure 3.14d). NMR analysis carried out in CDCl$_3$ 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 D$_2$O exhibited very few broad peaks representing PEG and PCL blocks only. These results suggest that coumarin-6 was located in the core of the micelles 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 for PLA and coumarin-6. Strong peak of PEG in D$_2$O was observed indicating that the location of PEG is in the corona of the micelles. Results from this study suggest that PB copolymers 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 (Figure 3.15). As the aqueous concentration of polymer was raised from 0.1 wt% to 5 wt%, two peaks (22 nm and 150 nm) were continuously shifted toward higher particle size. Moreover, a 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 (~5 µm) was also revealed. Increase in particle size and polydispersity as a function of concentration clearly indicate the involvement of a micellar aggregation mechanism.

Viscosity measurement

Table 3.3 describes the kinematic viscosity of 15 wt% aqueous gelling solutions of different block copolymers at various temperatures ranging from 5 to 25 °C. Kinematic viscosities of polymer solutions accelerated with rise in temperature. Interestingly, at any given temperature, an increase in molecular weight of the block copolymers exhibited higher viscosity (TB-1 and TB-2 or PB-1 and PB-2).

Also, the viscosities of TB-2 and PB-5 (hydrophobic polymers) were considerably higher relative to PB-1 and PB-4 (hydrophilic polymers), respectively. We speculate that the 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 a noticeable effect on viscosity. Hence, kinematic viscosity of PB-1 solution was significantly higher compared to PB-5 aqueous solution.
Figure 3.12: Effect of block copolymer composition on in vitro release of IgG from thermosensitive gels (20 wt%).
Figure 3.13: Effect of polymer concentrations (15, 20 and 25 wt%) on *in vitro* release of IgG from PB-1 thermosensitive gels.
Table 3.2: Coefficient of determination ($R^2$) for various kinetic models for *in vitro* release of IgG.

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Figure 3.14: $^1$H-NMR of PB-1 and coumarin-6 in (a) CDCl$_3$ and (b) D$_2$O, and PB-5 and coumarin-6 in (c) CDCl$_3$ and (d) D$_2$O.
Hypothesis for gelation mechanisms

From the observed results of $^1$H-NMR, viscosity and micelle size analysis, we propose two different mechanisms of sol-gel transition for different types of block copolymers related to, (i) the 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) the hydrophilic segments at the termini (A-B-C-B-A (PB-4 and PB-5)). According to our 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 segments. Therefore, as described in Figure 3.16, interaction of PEG with water molecules dominated at low temperature (4 °C) allowing the polymer to solubilize (Figure 3.16a). In contrast, elevation in temperature causes polymer aggregation and initiates the process of micellization. Therefore, it is possible to solubilize a hydrophobic dye (coumarin-6) in an aqueous solution of PB-1 copolymers. B-A-B or C-B-A-B-C copolymers possess hydrophobic termini and hence behave differently in aqueous solution relative to A-B-C-B-A types of copolymers. In the case of B-A-B or C-B-A-B-C copolymers, during micellization polymer molecules can serve as intermicellar bridges, where the hydrophobic ends of the polymer are diffused in the core of different micelles (Figure 3.16b). 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 3.3). 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 an increase in polymer concentration (Figure 3.15). At CGT, intermicellar bridges and micellar aggregation may be sufficient enough to form the opaque hydrogel (Figure 3.16c). Further heating of the heterogeneous opaque gel results in polymer
precipitation. This may be due to strong hydrophobic interactions between PCL or PCL-PLA chains that can significantly overcome weaker hydrophilic interactions (hydrogen bonds) between PEG and water. This phenomenon may eventually dehydrate the PCL or PCL-PLA core leading to collapse of hydrogel structure (Figure 3.16d).

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 lack 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 the opaque hydrogel. Further heating of the gelling solution (above CPT) may lead to dominate hydrophobic interactions causing dehydrated micelle cores which eventually result in polymer precipitation.
Figure 3.15: Estimation of PB-1 copolymer micelles at various concentrations i.e., 0.1, 0.5, 2 and 5 wt%.
Table 3.3: Viscosity of thermosensitive gelling solutions (15 wt%) at various temperatures.

<table>
<thead>
<tr>
<th>Block copolymers</th>
<th>Viscosity (cp) at various temperature</th>
<th>5°C</th>
<th>10°C</th>
<th>15°C</th>
<th>20°C</th>
<th>25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB-1</td>
<td></td>
<td>2.45 ± 0.07</td>
<td>2.75 ± 0.02</td>
<td>3.30 ± 0.07</td>
<td>3.76 ± 0.12</td>
<td>-</td>
</tr>
<tr>
<td>TB-2</td>
<td></td>
<td>3.31 ± 0.11</td>
<td>3.95 ± 0.09</td>
<td>4.39 ± 0.06</td>
<td>5.30 ± 0.07</td>
<td>-</td>
</tr>
<tr>
<td>PB-1</td>
<td></td>
<td>2.85 ± 0.05</td>
<td>3.02 ± 0.09</td>
<td>3.40 ± 0.04</td>
<td>3.95 ± 0.02</td>
<td>-</td>
</tr>
<tr>
<td>PB-2</td>
<td></td>
<td>3.56 ± 0.05</td>
<td>4.02 ± 0.14</td>
<td>4.60 ± 0.02</td>
<td>5.85 ± 0.08</td>
<td>-</td>
</tr>
<tr>
<td>PB-4</td>
<td></td>
<td>1.92 ± 0.03</td>
<td>2.16 ± 0.05</td>
<td>2.53 ± 0.08</td>
<td>2.76 ± 0.04</td>
<td>3.16 ± 0.12</td>
</tr>
<tr>
<td>PB-5</td>
<td></td>
<td>2.07 ± 0.08</td>
<td>2.22 ± 0.06</td>
<td>2.63 ± 0.05</td>
<td>2.97 ± 0.08</td>
<td>3.38 ± 0.11</td>
</tr>
</tbody>
</table>
Figure 3.16: 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-assembles to form micelles, (c) 37 °C, formation of intermicellar bridges resulting in gel formation, and (d) 50 °C, dehydration of PCL segments to water phase resulting in precipitation.
Conclusion

Compositions of PCL-PEG-PCL (B-A-B), PLA-PCL-PEG-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 an injectable *in situ* hydrogel forming depot for controlled ocular protein delivery. PB copolymers exhibit significantly reduced crystallinity. It is anticipated that biodegradability of these novel copolymers is significantly improved relative to TB copolymers. PB copolymers with A-B-C-B-A block arrangements are easy to handle at room temperature. Cell viability and biocompatibility studies confirmed that PB copolymers may be considered safe biomaterials for ocular delivery. More importantly, PB copolymers also exhibit significant sustained release of IgG relative to TB copolymers. These outcomes clearly suggest that a PB copolymer based controlled drug delivery system may serve as a promising platform for back of the eye complications.
CHAPTER 4
SUSTAINED DELIVERY OF PROTEINS EMPLOYING NOVEL PENTABLOCK COPOLYMER BASED NANOPARTICLULATE SYSTEM FOR THE TREATMENT OF POSTERIOR SEGMENT OCULAR DISEASES

Rationale

A considerable amount of research has been carried out for the development of peptide- or protein-encapsulated, sustained delivery formulations for the treatment of posterior segment diseases such as wet AMD and DR. However, physical and chemical instability, and rapid enzymatic degradation of protein therapeutics are few of the many challenges encountered by formulation scientists during the development of controlled release formulation. To date, biodegradable micro- and nanoparticles (NPs) are proven to be the most promising carrier systems that provide peptide/protein asylum against catalytic enzymes, allowing improvement in their biological half-lives.

Among all biomaterials, biodegradable polymers such as PLA [188], PCL-PEG-PCL [189], and PLGA [190] have been extensively investigated for the development of peptide- or protein-encapsulated NPs. It is widely reported that protein/peptide can undergo a loss of biological activity during formulation, storage and/or release [191-194]. Presence of hydrophobic/hydrophilic interface [195], reduction in pH during polymer degradation [196], and polymer degradation product-induced acylation processes [183, 197] are potential sources of irreversible aggregation or inactivation of therapeutic proteins inside the PLA, PLGA, PCL-PLA-PCL and PLA-PEG-PLA based delivery systems. It is imperative to note that any change in protein/peptide structure, either physically or chemically, may cause toxicity or immunogenicity and inactivity (loss of pharmacological effect). The consecutive response to
antibody directs safety concerns and thus restricts efficacy of subsequent applications [198]. Therefore, there is an urgent need to develop a biodegradable sustained-release system, which does not compromise protein stability during release. In addition, sustained release of therapeutic agents for longer durations can eliminate repeated intravitreal injections.

PB copolymers with thermosensitive properties, studied in chapter 3 exhibited sustained release of IgG up to only ~15-20 days. Sustained release for lower duration and significantly higher burst release exhibited by thermosensitive gels are unfavorable. Hence in this study, we have investigated PB copolymers with block arrangements of PLA-PCL-PEG-PCL-PLA and PGA-PCL-PEG-PCL-PGA for the development of protein-loaded NPs which can sustain drug release for significantly longer duration of time (~60 days). We have synthesized and characterized PB copolymer variants to investigate effects of molecular weight, isomerism and polymer composition on various parameters such as entrapment efficiency, drug loading, and in vitro drug release profiling. Human immunoglobulin G (IgG, 150 kDa), a full length antibody was utilized as model protein which is very similar to bevacizumab.

**Materials and methods**

**Materials**

PEG (4 kDa), stannous octoate, ε-caprolactone, polyvinylalcohol (PVA), lipopolysaccharide were procured from Sigma-Aldarich (St. Louis, MO; USA). L-lactide and D,L-lactide were purchased from Acros organics (Morris Plains, NJ; USA). Micro-BCA™ was obtained from Fisher Scientific. Mouse TNF-α, IL-6 and IL-1β (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. ARPE-19 and RAW-264.7 cells were procured from the American type culture collection (ATCC).

**Methods**

*Synthesis of TB and PB copolymers*

The TB and PB copolymers were synthesized by ring-opening bulk copolymerization [176]. In brief, PCL-PEG-PCL copolymers were synthesized by copolymerization of ε-caprolactone on the hydroxyl ends of PEG (4 kDa). In this reaction, PEG was utilized as macroinitiator and stannous octoate (0.5 wt%) as a catalyst. To synthesize PCL-PEG-PCL, a pre-determined amount of PEG was vacuum-dried for 4 h followed by addition of ε-caprolactone and catalyst. Reaction was carried out in a closed vessel under nitrogen, at 130 °C for 36 h. The reaction mixture was solubilized in DCM followed by precipitation in ice-cold diethyl ether. The precipitated polymer was vacuum-dried to remove residual solvent and characterized to evaluate the reaction yield. The structure and molecular weight of TB copolymers were confirmed by $^1\text{H}$-NMR and GPC.

In order to prepare PB copolymers, a predetermined amount of TB copolymer was utilized as macroinitiator and stannous octoate (0.5 wt%) as catalyst. For the synthesis of PB-A and PB-D, L-lactide was polymerized on the hydroxyl ends of TB copolymer. Similarly, PB-B/PB-E, and PB-C/PB-F were synthesized by polymerizing D,L-lactide and glycolide, respectively. For the synthesis of PB-A, PB-B, PB-D and PB-E, the reaction was carried out at 130 °C for 36 h. However, to synthesis of PB-C and PB-F, reaction was performed at 200 °C for 24 h.
**Figure 4.1:** Synthesis scheme for TB-A, TB-B, PB-A, PB-B, PB-D and PB-E.
Figure 4.2: Synthesis scheme for PB-C and PB-F. Note: For synthesis of PB-C and PB-F step 1 was similar as described in Figure 4.1.
In order to remove catalyst and unreacted monomers, the reaction mixture was dissolved in DCM and precipitated by addition of ice-cold diethyl ether. Polymers were vacuum-dried and characterized for their structure and polydispersity by employing $^{1}$H-NMR and GPC as analytical techniques. Purified polymers were stored at -20 °C until further use. Reaction scheme for the synthesis of TB-A, TB-B, PB-A, PB-B, PB-D and PB-E are described in Figure 4.1 whereas for the synthesis of PB-C/PB-F are described in Figure 4.2.

**Characterization of polymers**

Polymers were characterized for purity, molecular weight and polydispersity by $^{1}$H-NMR and GPC. Polymers were further evaluated by powder XRD to examine their crystalline nature.

$^{1}$H-NMR

To perform $^{1}$H-NMR spectroscopy, polymeric material was dissolved in CDCl$_3$ and analyzed on a Varian-400 MHz NMR spectrometer. Purity and molecular weight ($M_n$) of the polymers were confirmed from the $^{1}$H-NMR spectrum.

**GPC**

To further confirm purity, molecular weight and polydispersity, polymeric samples were analyzed via GPC equipped with refractive index detector (Waters 410). Briefly, 5 mg of polymeric material was dissolved in tetrahydrofuran (THF). THF was utilized as eluting solvent at the flow rate of 1 mL/min whereas separation was carried out on Styragel HR-3 column. Polystyrene samples with narrow molecular weight distribution were utilized as standards.
X-ray diffraction (XRD) analysis of copolymers

Samples were analyzed for diffraction patterns to understand the effects of polymer composition (TB vs PB), molecular weight, and block types (P(L)LA, P(DL)LA and PGA) on the crystallinity of copolymers. MiniFlex automated X-ray diffractometer (Rigaku, The Woodlands, Texas) with Ni-filtered Cu-κα radiation (30 kV and 15 mA) was employed to study the diffraction patterns. XRD analysis was carried out at room temperature.

In vitro cytotoxicity studies

Cell culture

RAW-264.7 cells were cultured and maintained in Dubecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 100 mg/L of streptomycin and 100 U/L of penicillin. Human retinal pigment epithelial cells (ARPE-19) were cultured and maintained according to the protocol reported from our lab [199]. In brief, ARPE-19 cells were cultured in DMEM/F-12 medium containing 10% heat-inactivated fetal bovine serum (FBS), 29 mM of sodium bicarbonate, 15 mM of HEPES, 100 mg/L of streptomycin and 100 U/L of penicillin. Both cell lines were maintained in humidified atmosphere at 37 °C and 5% CO₂.

Lactate Dehydrogenase (LDH) assay

The cytotoxicity of block copolymers were evaluated according to previously published protocol with minor modifications [200]. Briefly, different concentrations (1-20 mg/mL) of TB and PB copolymers were prepared in acetonitrile (ACN). A 100 μL of solutions was aliquoted in each well of 96-well cell culture plates. In order to evaporate ACN and to sterilize block copolymers, cell culture plates were exposed overnight under UV light (laminar flow). Once the ACN is evaporated, 1.0 x 10⁴ of RAW 264.7 cells were seeded in each well and incubated for 48 h at 37 °C and 5% CO₂ in humidified atmosphere. Followed by
appropriate incubation, cell supernatant were analyzed for the levels of LDH by LDH detection kit. LDH assay was performed according to the supplier’s protocol. Samples were analyzed at 450 nm by 96-well plate reader. Amount of released LDH is directly proportional to cytotoxicity of the polymers. In this study, more than 10% of LDH release was considered as cytotoxic. To evaluate toxicity of block copolymers on retinal cell line, the similar experiment was performed with ARPE-19 cells. LDH release (%) was calculated by employing following equation,

\[
LDH\ release(\%) = \frac{Abs.\ of\ Sample - Abs.\ of\ negative\ control}{Abs.\ of\ positive\ control - Abs.\ of\ negative\ control} \times 100 \quad \ldots \text{Eq. 4.1}
\]

**MTS assay**

In order to confirm safety of PB copolymers, *in vitro* cell viability (MTS) assay was performed. MTS assay was carried out according to previously published protocol with minor modifications [185]. Different concentrations of block copolymer solutions were prepared, aliquoted and sterilized according the procedure mentioned in previous section. After sterilization, RAW-264.7 cells at the density of 1.0 \times 10^4 cells per well were seeded in 96-well plate. Cells were incubated for 48 h at 37 °C and 5% CO\(_2\) in humidified atmosphere. After incubation, cell culture medium was replaced with 100 µL of serum free medium containing 20 µL of MTS solution. Cells were further incubated at 37 °C and 5% CO\(_2\) for 4 h. Absorbance of each well was determined at 450 nm. Polymer concentrations at which more than 90% of cell viability was observed, were considered as non-toxic. Percent cell viability was estimated by following equation. The similar experiment was repeated with ARPE-19 cells.

\[
Cell\ viability\ (\%) = \frac{Abs.\ of\ Sample - Abs.\ of\ negative\ control}{Abs.\ of\ positive\ control - Abs.\ of\ negative\ control} \times 100 \quad \ldots \text{Eq. 4.2}
\]
**In vitro biocompatibility studies**

As described in previous section, different concentrations (1-20 mg/mL) of block copolymer solutions were prepared in acetonitrile. A 200 µL aliquot of polymer solution was added in each well of 48-well cell culture plate. These plates were incubated overnight under UV light (laminar flow) for sterilization as well as for evaporation of ACN. After sterilization, RAW-264.7 cells were plated at the cell density of 5.0 x 10⁴ per well. Cells were exposed to polymer for 48 h at 37 °C and 5% CO₂. After incubation, cell supernatant was analyzed for the quantification of three different cytokines, i.e., TNF-α, IL-6 and IL-1β. Levels of cytokines were estimated by ELISA method. ELISA was performed according to the manufacturer’s instruction. 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.

**Preparation of NPs**

NPs were prepared by W₁/O/W₂ double emulsion solvent evaporation method according to previously published protocol with minor modifications [201]. Briefly, predetermined amount of IgG was dissolved in water (1 mL) containing 50 µL of Tween-80 (W₁ phase) and 100 mg of respective block copolymer was dissolved in 4 mL of dichloromethane (DCM) comprising 50 µL of Span-20 (organic phase). W₁/O primary emulsion was prepared by drop-wise addition of W₁ phase in organic phase under constant sonication for 1 min at 4 W output. Immediately after sonication, W₁/O primary emulsion was added (drop-wise) in 20 mL of 2% PVA solution (W₂) and sonication was applied for 4 min at 5 W output. W₁/O/W₂ double emulsion was stirred for 30 min at room temperature followed by evaporation of DCM under vacuum. The resulting NPs were centrifuged at 20000 rpm for 30 min at 4 °C followed by two washing cycles with distilled deionized water. NPs were freeze-
dried in 5% mannitol solution and stored at -20 °C until further characterization. NPs were prepared utilizing three different drug to polymer ratios i.e., 1:10, 1:15 and 1:20. Freeze-dried NPs were characterized for particle size, EE, DL and *in vitro* drug release behavior.

**Characterization of NPs**

**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 angel utilizing Zeta sizer (Zetasizer Nano ZS, Malvern Instruments Ltd, Worcestershire, UK). All the samples were analyzed in triplicate and average particle size were reported in ± SD.

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

IgG-loaded freeze-dried NPs were examined for the estimation of DL and EE. EE was calculated by analyzing supernatants (collected during NP preparation) with Micro BCA™ protein estimation kit. To evaluate DL, 2 mg equivalent drug-encapsulated NPs were dissolved in 200 µL of DMSO. The resulting solution was subjected for protein estimation via UV absorbance spectroscopy. A standard curve of IgG ranging from 31.25 to 2000 µg/mL was prepared in DMSO. EE (%) and DL (%) were estimated according to the following equations.

EE (%) was calculated with eq. 4.3

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

…Eq. 4.3

DL (%) was calculated by eq. 4.4.

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

…Eq. 4.4
**In vitro release studies**

For *in vitro* drug release studies, 1 mg protein equivalent IgG-encapsulated NPs were dispersed in 1 mL of 0.1 M phosphate buffer saline (pH - 7.4). The NPs suspension was incubated in water bath maintained at 37 °C. In order to collect release samples, at pre-determined time intervals NPs suspension were centrifuged at 13000 rpm for 30 min and 4 °C. A 200 µL of clear supernatant was withdrawn for protein estimation and replaced with same volume of 0.1 M PBS. NPs where then resuspended and release was continued at 37 °C. *In vitro* release experiments were repeated in triplicates and mean value ± SD was expressed as cumulative % drug released with time.

**Stability estimation of IgG**

*Secondary structure analysis by circular dichroism (CD) spectroscopy*

To confirm the stability of secondary structure of released IgG, CD spectroscopy was employed as an analytical technique. CD analysis was performed using Jasco 720 spectropolarimeter at 25 °C. CD spectra were recorded at scanning speed of 5 nm/min between the range of 200 to 250 nm utilizing 1 cm cell, and 1 nm band width. CD measurements were described as molar ellipticity \([\theta]\). A CD spectrum of PBS was used as blank.

**Results and discussion**

In this study, we have prepared novel PB copolymers which are composed of FDA approved polymer blocks such as PEG, PCL, and PLA/PGA. Each block plays an important role such as presence of PEG helps to improve stability of NPs by reducing NP aggregation, and also prevents phagocytosis by macrophages resulting in improved half-life. PCL is a slowly degrading, semi-crystalline polymer which improves protein loading in NPs and also sustains drug release for longer duration of time. Such a slow degradation is also not
advantageous particularly for intravitreal injections. It is very important for formulation scientists to synchronize polymer degradation profile with the drug release profile in order to avoid accumulation of empty formulation in the limited space of the vitreous cavity. Previous reports suggest that poor degradation of PCL is attributed to its crystalline nature, hence reduction in the crystallinity of PCL may improve its hydrolytic and enzymatic degradation [202]. According to Huang, et al. conjugation of PLA/PGA to PCL chains can significantly reduce its crystallinity resulting in faster degradation of PCL [203].

**Synthesis and characterization of various TB and PB copolymers**

TB and PB copolymers were successfully synthesized by ring-opening bulk copolymerization of ε-caprolactone, and L-lactide/D,L-lactide/glycolide. In the first step, TB copolymers (PCL-PEG-PCL) with different molecular ratios of PEG/PCL were synthesized utilizing PEG (4kDa) as macroinitiator and stannous octoate as catalyst. Purified TB copolymers were used as macroinitiator for the synthesis of PB copolymers. Purity and molecular weight (Mn) of block copolymers were confirmed by \(^1\)H-NMR spectroscopy. As described in Figure 4.3, typical \(^1\)H-NMR characteristic peaks of PCL units were observed at 1.40, 1.65, 2.30 and 4.06 ppm representing methylene protons of -(CH\(_2\))\(_3\)-, -OCO-CH\(_2\)-, and -CH\(_2\)OOC-, respectively. A sharp proton peak observed at 3.65 ppm was attributed to methylene protons (-CH\(_2\)CH\(_2\)O-) of PEG.
**Figure 4.3:** $^1$H-NMR of TB-B (PCL$_{7000}$-PEG$_{4000}$-PCL$_{7000}$).
Figure 4.4: $^1$H-NMR of PB-A (PL(L)A$_{2000}$-PCL$_{5000}$-PEG$_{4000}$-PCL$_{5000}$-PL(L)A$_{2000}$).
Figure 4.5: Gel permeation chromatogram of PB-A copolymer.
Figure 4.6: XRD patterns of various TB and PB copolymers where (a) TB-A, PB-A, PB-B, and PB-C, (b) TB-B, PB-D, PB-E, and PB-F.
Table 4.1: List of TB and PB copolymers studied

<table>
<thead>
<tr>
<th>Code</th>
<th>Structures</th>
<th>PLA/PCL/PEG or PGA/PCL/PEG</th>
<th>Total Mn&lt;sup&gt;a&lt;/sup&gt; (theoretical)</th>
<th>Total Mn&lt;sup&gt;b&lt;/sup&gt; (calculated)</th>
<th>Total Mn&lt;sup&gt;c&lt;/sup&gt; (calculated)</th>
<th>Mw&lt;sup&gt;c&lt;/sup&gt; (GPC)</th>
<th>PD&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB-A</td>
<td>PCL&lt;sub&gt;5000&lt;/sub&gt;-PEG&lt;sub&gt;4000&lt;/sub&gt;-PCL&lt;sub&gt;5000&lt;/sub&gt;</td>
<td>2.5/1</td>
<td>14000</td>
<td>13350</td>
<td>10990</td>
<td>14520</td>
<td>1.32</td>
</tr>
<tr>
<td>TB-B</td>
<td>PCL&lt;sub&gt;7000&lt;/sub&gt;-PEG&lt;sub&gt;4000&lt;/sub&gt;-PCL&lt;sub&gt;7000&lt;/sub&gt;</td>
<td>3.5/1</td>
<td>18000</td>
<td>16650</td>
<td>14560</td>
<td>18800</td>
<td>1.29</td>
</tr>
<tr>
<td>PB-A</td>
<td>PL(L)A&lt;sub&gt;2000&lt;/sub&gt;-PCL&lt;sub&gt;5000&lt;/sub&gt;-PEG&lt;sub&gt;4000&lt;/sub&gt;-PCL&lt;sub&gt;5000&lt;/sub&gt;-PL(L)A&lt;sub&gt;2000&lt;/sub&gt;</td>
<td>1/2.5/1</td>
<td>18000</td>
<td>16950</td>
<td>14990</td>
<td>20800</td>
<td>1.39</td>
</tr>
<tr>
<td>PB-B</td>
<td>PL(DL)A&lt;sub&gt;2000&lt;/sub&gt;-PCL&lt;sub&gt;5000&lt;/sub&gt;-PEG&lt;sub&gt;4000&lt;/sub&gt;-PCL&lt;sub&gt;5000&lt;/sub&gt;-PL(DL)A&lt;sub&gt;2000&lt;/sub&gt;</td>
<td>1/2.5/1</td>
<td>18000</td>
<td>16880</td>
<td>13810</td>
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<tr>
<td>PB-C</td>
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<td>18000</td>
<td>17210</td>
<td>13450</td>
<td>19210</td>
<td>1.43</td>
</tr>
<tr>
<td>PB-D</td>
<td>PL(L)A&lt;sub&gt;3000&lt;/sub&gt;-PCL&lt;sub&gt;7000&lt;/sub&gt;-PEG&lt;sub&gt;4000&lt;/sub&gt;-PCL&lt;sub&gt;7000&lt;/sub&gt;-PL(L)A&lt;sub&gt;3000&lt;/sub&gt;</td>
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<td>21350</td>
<td>17640</td>
<td>23830</td>
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<td>PB-E</td>
<td>PL(DL)A&lt;sub&gt;3000&lt;/sub&gt;-PCL&lt;sub&gt;7000&lt;/sub&gt;-PEG&lt;sub&gt;4000&lt;/sub&gt;-PCL&lt;sub&gt;7000&lt;/sub&gt;-PL(DL)A&lt;sub&gt;3000&lt;/sub&gt;</td>
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<td>24000</td>
<td>21330</td>
<td>18210</td>
<td>24940</td>
<td>1.37</td>
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<tr>
<td>PB-F</td>
<td>PGA&lt;sub&gt;3000&lt;/sub&gt;-PCL&lt;sub&gt;7000&lt;/sub&gt;-PEG&lt;sub&gt;4000&lt;/sub&gt;-PCL&lt;sub&gt;7000&lt;/sub&gt;-PGA&lt;sub&gt;3000&lt;/sub&gt;</td>
<td>1.5/3.5/1</td>
<td>24000</td>
<td>21290</td>
<td>16180</td>
<td>23210</td>
<td>1.43</td>
</tr>
</tbody>
</table>

a. Theoretical value, calculated according to the feed ratio  
b. Calculated from <sup>1</sup>H-NMR results  
c. Determined by GPC analysis
The 	extsuperscript{1}H-NMR spectra of PB copolymers with PLA as terminals (PB-A, PB-B, PB-D and PB-E) exhibited two additional peaks at 1.50 (-CH\textsubscript{3}) and 5.17 (-CH-) ppm (Figure 4.4). However, PB-C and PB-E exhibited cluster of singlets between 4.6 to 4.9 ppm representing methylene protons (-CH\textsubscript{2}-) of PGA units (data not shown). The [EO]-[CL]-[LA] molar ratio of the final products were calculated from the integration values of PEG signal at 3.65 ppm, PCL signal at 2.30 ppm and PLA signal at 5.17 ppm. In case of PB-C and PB-F copolymers, proton signals between 4.6 to 4.9 ppm were utilized for the calculation of molar ratio.

In order to confirm the molecular weight and purity, all the block copolymers were further analyzed by GPC. Block copolymers exhibited a monodistributed molecular weight and absence of any other homopolymers such as PEG, PCL, PLA or PGA. A typical GPC chromatogram of PB-A copolymer is depicted in Figure 4.5. Moreover, calculated molecular weights were very close to the feed ratio, and polydispersity of block copolymers were well below 1.45 indicating narrow distribution of molecular weights. Table 4.1 represents theoretical molecular weights, calculated molecular weights (\textsuperscript{1}H-NMR and GPC) and polydispersity. The Mn values obtained from \textsuperscript{1}H-NMR were noticeably higher relative to the Mn values observed from GPC analysis. This observation may attributed to the difference in hydrodynamic diameter of block copolymers relative to parent homopolymers. As reported in Table 4.1, observed molecular weights were very similar to theoretical molecular weights. For the ease, in the following text, theoretical molecular weights will be mentioned instead of calculated molecular weights.

Reports published elsewhere described that covalent conjugation between PLA and PCL, significantly reduces the crystallinity of PCL [176, 204-207]. However, effects of various isomers of PLA (L or D,L) and also an effect of PGA on the crystallinity of PCL-PEG-PCL
tri-block copolymers are not reported, yet. Hence, we have analyzed TB and PB copolymers for their XRD patterns. PLA with D,L-lactide has amorphous structure whereas PLA with L-lactide is a semi-crystalline [202]. Both polymers may act differently to reduce the crystallinity of PCL. This may have direct effect on various formulation parameters such as EE, DL and *in vitro* release. Moreover, we have also evaluated the effect of PGA to reduce the crystallinity of PCL-PEG-PCL TB copolymers.

Figure 4.6 describes the XRD patterns of various TB and PB copolymers. As reported in Figure 4.6a, TB-A exhibited two strong characteristic crystalline peaks of PCL blocks at diffraction angles (2θ) of 21.5° and 23.8°. L-lactide containing PB copolymer (PB-A) exhibited reduced intensity of PCL peaks, which suggest that conjugation of PLA (L-lactide) has significantly diminished the crystallinity of TB-A. Noticeably, conjugation of D,L-lactide has further reduced the crystalline peaks of PCL blocks. These results may be attributed to the fact that PLA with L-lactide is more crystalline than PLA with D,L-lactide. Moreover, PLA with D,L-lactide have a random arrangement of L and D-lactide in their polymer chain which does not allow systemic arrangement of polymer chains, resulting in reduced crystallinity. Surprisingly, PB-C demonstrated amorphous structure suggesting that conjugation of PGA at the termini of TB-A have totally eliminated crystallinity of PCL. To further confirm this behavior, we have evaluated the solid states of TB-B, PB-D, PB-E and PB-F by XRD. Results described in Figure 4.6b demonstrated similar pattern, where PB-F with PGA a block was amorphous in nature. PLA with L-lactide reduced peak intensity of PCL up to some extent, while PLA with D,L-lactide has significantly diminished crystalline peaks.
**Figure 4.7**: *In vitro* cytotoxicity (LDH) assay of various block copolymers at different concentrations were performed on (a) RAW 264.7 and (b) ARPE-19 cells.
Figure 4.8: In vitro cell viability (MTS) assay of various block copolymers at different concentrations were performed on (a) RAW 264.7 and (b) ARPE-19 cells.
Figure 4.9: *In vitro* biocompatibility of block copolymers were evaluated by estimating the levels of (a) TNF-α, (b) IL-6 and (c) IL-1β in the supernatants of polymer treated RAW 264.7 cells.
**In vitro cytotoxicity studies**

**LDH assay**

Lactate dehydrogenase (LDH) is the cytosolic enzyme, secreted in the culture medium upon the rupture of cell membrane. As the cell membrane is the potential site for polymer-cell interaction, measuring the amount of LDH release has been the preferred way to estimate the membrane damage and hence the cytotoxicity of polymer. As described in Figure 4.7a (RAW-264.7 cells) and Figure 4.7b (ARPE-19), LDH release upon the exposure of block copolymers were less than 10% at any given concentrations and also comparable with negative control indicating negligible or no toxicity.

**MTS assay**

In order to confirm the results observed in cytotoxicity (LDH assay) studies, the safety of block copolymers were further evaluated by MTS assay. Block copolymers exhibited more than 90% cell viability upon exposure to various concentrations ranging from 1-20 mg/mL (Figures 4.8a and 4.8b). Moreover, results were comparable to the negative control indicating no toxicity of PB copolymers. Results observed in LDH and MTS assays clearly indicated that block copolymers are very safe for the intravitreal applications.

**In vitro biocompatibility studies**

It is also important to confirm that newly synthesized PB copolymers are not producing inflammatory responses upon intravitreal administration. *In vitro* assessment for release of cytokines upon exposure to polymer is a quick, cost effective and reliable technique to examine biocompatibility of polymers. RAW-264.7 cells, is a well-established *in vitro* cell culture model to study inflammatory responses of polymers intended for human applications. Results described in Figures 4.9a, 4.9b and 4.9c have demonstrated negligible release of TNFα, IL-6
and IL-1β upon 24 h exposure to different concentrations of block copolymers ranging from 1-20 mg/mL. Release of any cytokines was not significantly different to their respective negative controls indicating excellent biocompatibility of block copolymers for ocular applications.

**Characterization of NPs**

**Particle size**

IgG-loaded TB and PB NPs were prepared by the W₁/O/W₂ double emulsion solvent evaporation method. As described in Tables 4.2, 4.3 and 4.4, the size of NPs were between 285 nm to 330 nm. We did not observe any significant effect of polymer composition on the particle size. Moreover, in order to understand the effect of drug to polymer ratio on particle size, we have prepared NPs with three distinct drug to polymer ratios i.e., 1:10, 1:15 and 1:20. Interestingly, a change in drug to polymer ratio did not exhibit any noticeable effect on particle size. These results suggest that particle size is highly influenced by process parameters and not by polymer:drug ratio.

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

EE and DL are influenced by many parameters including copolymer composition (isomerism, molecular weight and polymer structure) and volumes of various phases (W₁, O and W₂). To understand the effect of copolymer compositions on DL and EE, we kept volumes of W₁, O and W₂ phases constant. In addition, we have also studied the effect of drug to polymer ratio on DL and EE. As described in Table 4.2, at respective drug to polymer ratios, TB-B NPs exhibited significantly higher DL and EE relative to TB-A NPs. PB-D, PB-E and PB-F (Table 4.3) with significantly higher molecular weight relative to PB-A, PB-B and PB-C (Table 4.4), respectively exhibited improved EE and DL.
Table 4.2: Characterization of NPs prepared TB-A and TB-B.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Drug/polymer ratio</th>
<th>Entrapment efficiency (%)</th>
<th>Loading (%)</th>
<th>Particle size(nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB-A</td>
<td>1:10</td>
<td>27.3 ± 2.2</td>
<td>3.6 ± 0.3</td>
<td>330 ± 20</td>
</tr>
<tr>
<td></td>
<td>1:15</td>
<td>28.2 ± 2.0</td>
<td>2.3 ± 0.1</td>
<td>320 ± 30</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>29.8 ± 1.9</td>
<td>2.0 ± 0.2</td>
<td>310 ± 30</td>
</tr>
<tr>
<td>TB-B</td>
<td>1:10</td>
<td>34.7 ± 3.2 *</td>
<td>4.1 ± 0.3 *</td>
<td>320 ± 20</td>
</tr>
<tr>
<td></td>
<td>1:15</td>
<td>33.7 ± 2.4 *</td>
<td>2.7 ± 0.2 *</td>
<td>300 ± 20</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>35.7 ± 2.8 *</td>
<td>2.6 ± 0.2 *</td>
<td>300 ± 20</td>
</tr>
</tbody>
</table>

*significantly different than respective ratios of TB-A NPs
Table 4.3: Characterization of NPs prepared PB-A, PB-B and PB-C.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Drug/polymer ratio</th>
<th>Entrapment efficiency (%)</th>
<th>Loading (%)</th>
<th>Particle size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB-A</td>
<td>1:10</td>
<td>46.8 ± 2.4 *</td>
<td>5.2 ± 0.3 *</td>
<td>320 ± 10</td>
</tr>
<tr>
<td></td>
<td>1:15</td>
<td>49.6 ± 3.1 *</td>
<td>3.8 ± 0.3 *</td>
<td>300 ± 20</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>47.7 ± 2.2 *</td>
<td>3.1 ± 0.2 *</td>
<td>300 ± 20</td>
</tr>
<tr>
<td>PB-B</td>
<td>1:10</td>
<td>51.5 ± 2.7 *</td>
<td>5.5 ± 0.4 *</td>
<td>300 ± 10</td>
</tr>
<tr>
<td></td>
<td>1:15</td>
<td>50.4 ± 4.1 *</td>
<td>4.2 ± 0.4 *</td>
<td>290 ± 20</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>53.5 ± 4.3 *</td>
<td>3.6 ± 0.3 *</td>
<td>290 ± 20</td>
</tr>
<tr>
<td>PB-C</td>
<td>1:10</td>
<td>39.7 ± 3.6</td>
<td>4.3 ± 0.5</td>
<td>310 ± 20</td>
</tr>
<tr>
<td></td>
<td>1:15</td>
<td>41.5 ± 4.2</td>
<td>3.2 ± 0.2</td>
<td>300 ± 20</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>40.3 ± 3.1</td>
<td>2.8 ± 0.1</td>
<td>290 ± 10</td>
</tr>
</tbody>
</table>

*significantly different than respective ratios of PB-C NPs
Table 4.4: Characterization of NPs prepared PB-D, PB-E and PB-F.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Drug/polymer ratio</th>
<th>Entrapment efficiency (%)</th>
<th>Loading (%)</th>
<th>Particle size(nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB-D</td>
<td>1:10</td>
<td>61.1 ± 4.2 *</td>
<td>5.7 ± 0.9 *</td>
<td>310 ± 20</td>
</tr>
<tr>
<td></td>
<td>1:15</td>
<td>61.8 ± 3.7 *</td>
<td>4.9 ± 0.3 *</td>
<td>300 ± 20</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>62.7 ± 4.0 *</td>
<td>3.8 ± 0.1 *</td>
<td>290 ± 10</td>
</tr>
<tr>
<td>PB-E</td>
<td>1:10</td>
<td>63.2 ± 2.2 *</td>
<td>5.8 ± 0.4 *</td>
<td>300 ± 20</td>
</tr>
<tr>
<td></td>
<td>1:15</td>
<td>64.9 ± 3.6 *</td>
<td>5.0 ± 0.3 *</td>
<td>300 ± 10</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>65.8 ± 3.8 *</td>
<td>4.3 ± 0.2 *</td>
<td>290 ± 10</td>
</tr>
<tr>
<td>PB-F</td>
<td>1:10</td>
<td>51.0 ± 3.0</td>
<td>5.0 ± 0.1</td>
<td>320 ± 30</td>
</tr>
<tr>
<td></td>
<td>1:15</td>
<td>52.4 ± 3.1</td>
<td>4.2 ± 0.2</td>
<td>310 ± 20</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>54.1 ± 3.7</td>
<td>3.2 ± 0.3</td>
<td>290 ± 20</td>
</tr>
</tbody>
</table>

*significantly different than respective ratios of PB-F NPs
It is important to note that higher molecular weights of block copolymers acquire longer polymer chain with significantly higher hydrophobicity relative to lower molecular weight block copolymers. Additionally, during the step of solvent evaporation, high hydrophobicity (PB-D, PB-E and PB-F) may allow faster polymer precipitation to form NPs preventing diffusion of IgG in the external aqueous (W₂) phase.

Interestingly, we have also observed significantly higher entrapment and loading efficiency in PLA based PB copolymers (PB-A, PB-B, PB-D and PB-E) relative to PGA based PB copolymers (PB-C and PB-F). These results may be attributed to the fact that PLA is more hydrophobic compare to PGA, hence its copolymers were also hydrophobic than that of PGA based copolymers. Due to higher hydrophilicity, PB-C and PB-F copolymers may have higher interaction with W₁ and W₂ phase which may allow IgG to escape from W₁ to W₂ phase (during NP preparation) resulting in lower EE and DL. In contrast, PLA based PB copolymers may have inhibited or reduced the diffusion of IgG from W₁ to W₂ phase ensuring improved DL and EE.

**In vitro release studies**

*In vitro* drug release studies were performed to understand the effects of isomerism (P(L)LA vs P(D,L)LA), molecular weight and polymer composition. IgG-loaded NPs with drug to polymer ratio of 1:10 were utilized for release study for all polymer compositions. *In vitro* release profiles of IgG from TB-A and TB-B NPs are compared in Figure 4.10. Both NPs demonstrated bi-phasic release profile i.e., initial burst release phase followed by a phase of sustained release. Noticeably, TB-A NPs exhibited significantly higher burst release (~64%) relative to TB-B NPs (~55%). In later stage, TB-A NPs displayed ~95% of IgG release in first 15 days whereas TB-B NPs released ~87% of IgG during the duration.
To evaluate the release of IgG from TB and PB NPs, and also to compare the effect of PGA and PLA based copolymers, we have compared release profiles of IgG from PB-A, PB-B, PB-C and TB-A NPs (Figure 4.11). Our results demonstrated that TB-A NPs exhibited significantly higher burst release (~64%) than any of the PB NPs (~41-49%). Moreover, PB NPs prolonged the IgG release for more than 27 days, whereas TB-A NPs sustained it for only 15 days. In addition, we have observed a noticeable effect of polymer structure on the burst release as well as on the duration of release. Surprisingly, burst release observed for PB-C (~49%) and PB-A (~48%) NPs were significantly higher compared to PB-B (~41%). PB-C NPs released ~93% of IgG over the period of 27 days, whereas PB-A (~91%) and PB-B (~90%) NPs prolonged the release up to 31 and 35 days, respectively.

As described earlier, PGA based PB-C copolymer is relatively hydrophilic compared to the PLA based PB-A and PB-B copolymers. It is anticipated that PB-C NPs may have higher affinity for protein molecules, hence these NPs may have higher amount of surface adsorbed drug. Moreover, being hydrophilic, PB-C NPs may become easily hydrated and allow easy diffusion of water molecules into the polymer matrix. Both the larger amount of surface adsorbed drug and higher affinity towards water may simultaneously contribute to the higher burst release and shorter sustained release period. We have also studied the effect of isomerism on drug release by comparing the release profiles of PB-A and PB-B copolymers. PB-A copolymers exhibited higher burst release with shorter sustained release phase relative to PB-B copolymers. As observed in XRD studies, PB-A copolymer is more crystalline than PB-B copolymer. In other words, polymer chains in PB-A NPs maybe in more ordered arrangement than in the PB-B NPs. Due to the ordered arrangements, during the preparation of NPs, the highly crystalline materials may form channels. Numbers and length of channels in PB-A NPs
might be higher than that of PB-B NPs. Water molecules may easily diffuse into the NPs via these channels facilitating the release of IgG. This phenomenon may also have contributed to the higher burst release and faster rate of release of IgG from PB-A NPs relative to PB-B NPs.

In order to support this reasoning, we have studied three more PB NPs i.e., PB-D, PB-E and PB-F. As described in Figure 4.12, PB-F exhibited ~92% of IgG release within 52 days with the burst release of ~43%. PB-D and PB-E demonstrated ~84% and ~87% of IgG release over the period of 55 and 59 days, respectively. However, all the PB NPs exhibited enhanced sustained release and a lower burst release compared to TB-B NPs. These results clearly indicated that PB-D, PB-E and PB-F NPs followed a similar trend to PB-A, PB-B and PB-C NPs.

To understand the effect of molecular weight on burst release as well as duration of release, we compared release profiles of PB-A and PB-D NPs (Figure 4.13a), PB-B and PB-E NPs (Figure 4.13b), and PB-C and PB-F NPs (Figure 4.13c). As discussed earlier, an increase in molecular weight of hydrophobic segments (PCL-PLA) also enhances hydrophobicity and polymer chain length. Hydrophobic copolymers may have less affinity with water and hence restrict the hydration of NPs. This may have resulted in less diffusion of water in NPs leading to the prolonged release of IgG. Moreover, longer chain length of polymers may have increased the mesh-like structure and reduced the effective porosity of NPs resulting in longer path for diffusion of IgG from NPs. In addition, reports published elsewhere suggest that with larger molecular weights, there is a slower rate of polymer degradation [208]. Therefore, PB-D, PB-E and PB-F may also degrade more slowly compared to PB-A, PB-B and PB-C, respectively, resulting in a slower rate of IgG release.
Figure 4.10: *In vitro* release of IgG from NPs prepared with TB-A and TB-B block copolymers.
Figure 4.11: In vitro release of IgG from NPs prepared with TB-A, PB-A, PB-B, and PB-C block copolymers.
Figure 4.12: *In vitro* release of IgG from NPs prepared with TB-B, PB-D, PB-E, and PB-F block copolymers.
Figure 4.13: Effect of molecular weights of block copolymers on \textit{in vitro} release of IgG (a) PB-A and PB-D NPs, (b) PB-B and PB-E NPs, and (c) PB-C and PB-F NPs.
Figure 4.14: CD spectrum of released IgG from PB-E NPs after 15 days.
Secondary structure stability estimation of IgG

It is very important for protein therapeutics to maintain their structural conformation to exert pharmacological actions. CD spectroscopy is a very sensitive analytical technique utilized for the estimation of secondary, and to some extent tertiary, structures of proteins. The method provides α-helix and β-sheet conformational information and also able to detect minor changes in protein conformation. Therefore, we have employed CD spectroscopy to study the conformational stability of released IgG and compared it with the CD spectrum of native IgG. Results reported in Figure 4.14 exhibited λ minima of released IgG at 218 nm which is similar to the λ minima of native IgG. Moreover, the entire spectrum of released IgG ranging from 200 nm to 250 nm was identical to native IgG suggesting retention of conformation of IgG during the NPs preparation and even after the release from NPs. Previous reports suggest that PLA and/or PGA based copolymer produce large molar mass of lactic acid and/or glycolic acid [183, 197]. These degradation products can stimulate hydrolytic degradation of protein therapeutics. The retention of protein stability in PB NPs may be attributed to lower molar masses of PLA or PGA blocks which produce lower amounts of lactic or glycolic acid, reducing the possibility of protein degradation.

Conclusion

We have successfully synthesized and characterized novel PB copolymers with different block ratios of PEG/PCL/PLA or PEG/PCL/PGA. These PB copolymers were studied for the development of protein-loaded NPs in the treatment of posterior segment diseases such as wet AMD and DR. Our results demonstrated that crystallinity of PB copolymers can be easily modulated by changing the ratios of PLA/PCL or PGA/PCL blocks and also by utilizing different isomers of PLA (L or D,L). Moreover, results exhibited that molecular weight,
crystallinity and copolymer composition have significant effects on EE, DL and *in vitro* release kinetics. PB copolymers composed of PLA with D,L-lactide exhibited higher EE and slower release relative to PB copolymers comprising PLA (L-lactide) or PGA. The stability of released IgG was established by CD spectroscopy. In addition, cytotoxicity, cell viability and *in vitro* inflammatory studies confirmed that PB copolymers are excellent biomaterials for the development of protein-loaded, sustained delivery formulations for the treatment of posterior segment ocular diseases.
CHAPTER 5

TAILOR-MADE PENTABLOCK COPOLYMER BASED COMPOSITE FORMULATION FOR SUSTAINED OCULAR DELIVERY OF PROTEIN THERAPEUTICS

Rationale

Various biodegradable polymeric NP formulations have been extensively investigated for controlled delivery of protein therapeutics. Biodegradable polymers such as PCL, PLA, PGA and PEG have been comprehensively studied for the preparation of protein-encapsulated NPs. Current challenges in the development of protein-encapsulated NPs are to ensure protein stability during the NP preparation and drug release. In addition, the second major problem with any NP formulation is higher burst release (initial rapid drug release phase).

The objective of this research is to synthesize and evaluate novel tailor-made PB copolymers for the controlled and non-invasive delivery of macromolecules in the treatment of posterior segment diseases. We have synthesized three PB copolymers by sequential ring-opening polymerization either for the preparation of protein-loaded NPs (two PB copolymers) or thermosensitive gel (one PB copolymer). Different ratios and molecular weights of each block (PGA, PEG, PLA and PCL) were selected for synthesis to optimize the release profile of FITC-BSA, IgG and bevacizumab from NPs. We have hypothesized that release of protein therapeutics from NPs is affected by their hydrodynamic diameter. Therefore, in this study we have examined effect of hydrodynamic diameter of proteins on EE and in vitro drug release. We have characterized NPs for particle size, polydispersity, EE, DL and in vitro release studies.

As described earlier, PB thermosensitive gel (chapter 3) and PB NPs (chapter 4) exhibited significantly higher burst release and relatively shorter duration of sustained release. In many of the cases, burst release (~25-45%) can exhibit dose dependent toxicity. Moreover,
loss of ~25-45% of dose in first two days can significantly reduce the duration of release. So in order to eliminate burst release phase and to achieve continuous zero-order drug release, we have prepared and characterized a novel composite formulation comprised of protein-loaded NPs suspended thermosensitive gelling solution. A composite formulation also offers an additional advantage to eliminate any possibility of floater effect which may arise during the intravitreal injection of NPs alone. Stability of released protein was also confirmed by CD spectroscopy and in vitro biological assays.

**Materials and methods**

**Materials**

PEG (1 kDa and 4 kDa), methoxy-PEG (550 Da), stannous octoate, ε-caprolactone, poly (vinyl alcohol) (PVA), lipopolysaccharide were procured from Sigma-Aldrich (St. Louis, MO; USA). L-lactide and hexamethylene diisocyanate (HMDI) were purchased from Acros organics (Morris Plains, NJ; USA). Micro-BCA™ was obtained from Fisher scientific. Mouse TNF-α, IL-6 and IL-1β (Ready-Set-Go) ELISA kits were purchased from eBioscience Inc. Lactate dehydrogenase estimation kit and CellTiter 96® AQ®ueous 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 PB copolymers**

Novel PB copolymers, PGA-PCL-PEG-PCL-PGA (PB-A), PLA-PCL-PEG-PCL-PLA (PB-B) and PEG-PCL-PLA-PCL-PEG (PB-C) were synthesized by ring-opening bulk polymerization method [176]. PB copolymers for the preparation of NPs i.e., PB-A and PB-B were synthesized in two steps by sequential ring-opening polymerization. PEG (1 kDa and 4
kDa) was utilized as macroinitiator and stannous octoate as a catalyst. In the first step, TB copolymer PCL-PEG-PCL (Figure 5.1, step 1) was synthesized by polymerization of \( \varepsilon \)-caprolactone on two open hydroxyl ends of PEG. In brief, PEG was dissolved in anhydrous toluene followed by distillation to remove residual moisture. \( \varepsilon \)-caprolactone and stannous octoate (0.5% w/w) were added to anhydrous PEG and temperature was raised to 130 °C. After 24 h, reaction mixture was dissolved in methylene chloride followed by precipitation in cold petroleum ether. The precipitated polymer was filtered and dried for 24 h under vacuum at room temperature. In the second step, the PCL-PEG-PCL TB copolymer was reacted with glycolide and L-lactide to prepare the PB-A (Figure 5.1, step 2) and PB-B (Figure 5.2) copolymers, respectively. The TB copolymer and glycolide/L-lactide were added in round bottom flask and temperature was raised to 130 °C under inert atmosphere. To this, stannous octoate (0.5% w/w) was added and reaction was allowed to run for 24 h. PB copolymer was purified by cold ether precipitation method described in the first step. The polymer was dried under vacuum and stored at -20 °C until further use.
Figure 5.1: Synthesis scheme for PB-A (PGA-PCL-PEG-PCL-PGA) copolymer.

Figure 5.2: Synthesis scheme for PB-B (PLA-PCL-PEG-PCL-PLA) copolymer.
Figure 5.3: Synthesis scheme for PB-C (PEG-PCL-PLA-PCL-PEG) copolymer.
For the synthesis of thermosensitive gelling polymer, TB copolymer (mPEG-PCL-PLA) was synthesized by ring-opening bulk copolymerization as described above (Figure 5.3). Firstly, ε-caprolactone was polymerized at the hydroxyl terminal of mPEG (550 Da) followed by polymerization of L-lactide. Resulting TB copolymers were coupled utilizing HMDI as a linker. Coupling reaction was carried out at 70 °C for 8 h. Polymers were purified by cold ether precipitation followed by drying under vacuum and stored at -20 °C.

**Characterization of polymers**

The resulting polymers were characterized for molecular weight and purity by $^1$H NMR spectroscopy and gel permeation chromatography (GPC).

$^1$H-NMR analysis

To perform $^1$H-NMR spectroscopy, polymeric materials were dissolved in CDCl$_3$ and spectra were recorded with Varian-400 NMR instrument. Purity and molecular weight ($M_n$) were calculated from the $^1$H-NMR spectra.

GPC analysis

Purity, molecular weights and polydispersity of PB copolymers were further confirmed by GPC analysis. Polymeric samples were analyzed with refractive index detector (Waters 410). Briefly, samples were prepared by dissolving 5 mg of polymeric material in tetrahydrofuran (THF) whereas THF was utilized as eluting agent at the flow rate of 1 mL/min. Separation was carried out on Styrage HR-3 column and polystyrene samples with narrow molecular weight distribution were utilized as standards.
In vitro cytotoxicity studies

Cell culture

Human retinal pigment epithelial cells (ARPE-19) were cultured and maintained according to the previously published protocol from our lab [199]. In brief, cells were cultured in Dulbecco's modified Eagle medium (DMEM)/F-12 medium containing 10% fetal bovine serum (FBS), 15 mM of HEPES, 29 mM of sodium bicarbonate, 100 U/L of penicillin and 100 mg/L of streptomycin. A mouse macrophage cell line, RAW-264.7 was procured from ATCC. RAW-264.7 cells were cultured and maintained in DMEM supplemented with 10% FBS, 100 U/L of penicillin and 100 mg/L of streptomycin. The Statens Serum Institut rabbit corneal (SIRC) cell line was purchased from ATCC at passage 400 and used between passages 410 - 425. During the study and maintenance period cells were supplemented with cell culture media composed of MEM containing 10% FBS, lactalbumin, HEPES, sodium bicarbonate, 100 U/L of penicillin and 100 mg/L of streptomycin. Human conjunctival epithelial cells (HCEC) were maintained in cell culture flask containing MEM Earle’s BSS medium supplemented by 10% FBS, 100 U/L of penicillin, 100 mg/L of streptomycin, 29 mM of sodium bicarbonate, and 2 mM L-glutamine. Choroid-retinal endothelial cells (RF/6A cells) were procured from ATCC. Briefly, cells were cultured and maintained in cell culture medium composed of RPMI-1640 comprising 10% FBS, 100 U/L of penicillin and 100 mg/L of streptomycin [209]. All five cell lines were maintained in humidified atmosphere at 37 °C and 5% CO2.

Lactate Dehydrogenase (LDH) assay

Previously published protocol with minor modification was employed to evaluate the cytotoxicity of PB copolymers [210]. Briefly, 10 mg/mL of PB copolymers (PB-A and PB-B) were dissolved in ACN and 100 µL of these solutions were aliquoted in each well of 96-well
cell culture plates. Plates were exposed overnight under UV light (laminar flow) for the sterilization of polymer and evaporation of ACN. ARPE-19 cells at the density of $1.0 \times 10^4$ were seeded in each well and incubated at $37^\circ C$, 5% CO$_2$ in humidified atmosphere for 48 h. After completion of incubation period, cell supernatant were analyzed for the quantification of LDH. According to supplier’s protocol, levels of LDH were analyzed utilizing a LDH detection kit. Absorbance of each well was estimated at 450 nm by 96-well plate reader. More than 10% of LDH release was considered as cytotoxic. To evaluate the cytotoxicity of block copolymers on conjunctiva, cornea and macrophages, the similar experiment was performed with HCEC, SIRC and RAW-264.7 cells. LDH release (%) was calculated with following equation,

$$LDH_{\text{release}}(\%) = \frac{\text{Abs. of Sample} - \text{Abs. of negative control}}{\text{Abs. of positive control} - \text{Abs. of negative control}} \times 100 \quad \cdots \text{Eq. 5.1}$$

**MTS assay**

Safety of PB copolymers was further established by performing in vitro cell viability assay (MTS assay) [211]. MTS assay was performed according to previously reported protocol with minor changes. As described earlier, PB copolymer solutions at the concentration of 10 mg/mL were prepared, aliquoted and sterilized. After sterilization, ARPE-19 cells were seeded in each well of 96-well plate at the cell density of $1.0 \times 10^4$, and incubated at $37^\circ C$ and 5% CO$_2$ in humidified atmosphere for 48 h. At the end of incubation period, cell culture medium was aspirated and cells were incubated for 4 h ($37^\circ C$ and 5% CO$_2$) in presence of 100 µL of serum free medium containing 20 µL of MTS solution. Absorbance of each well was estimated at 450 nm. The similar experiment was repeated with other ocular and macrophage cell line such as HCEC, SIRC and RAW-264.7 cells. Percent cell viability was calculated by the following equation.
Cell viability(%) = \frac{\text{Abs. of Sample} - \text{Abs. of negative control}}{\text{Abs. of positive control} - \text{Abs. of negative control}} \times 100 \quad \ldots \text{Eq. 5.2}

In this study, PB copolymers which exhibited more than 90% of cell viability were considered non-toxic for ocular applications.

**In vitro biocompatibility studies**

PB copolymers were dissolved in ACN at the concentration of 10 mg/mL and 200 µL of these solutions were aliquoted in each well of 48-well cell culture plates. Cell culture plates were incubated overnight under UV lights (laminar flow) for the evaporation of ACN and sterilization of resulting polymer film. After sterilization, RAW-264.7 cells (5.0 x 10⁴) were seeded in each well of cell culture plate and incubated for 24 h at 37 °C and 5% CO₂. After 24 h, cell supernatants were analyzed for the presence of cytokines such as TNF-α, IL-6 and IL-1β. Lipopolysaccharide (LPS) was utilized as positive control whereas cells without treatment were considered as negative control. Levels of cytokines were quantified by ELISA method which was performed according to supplier’s instructions. Standard 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.

**Preparation of NPs**

IgG-loaded PB NPs were prepared by W₁/O/W₂ double emulsion solvent evaporation method [212]. Briefly, predetermined quantity of IgG (10 mg) was dissolved in 0.1 M phosphate buffer saline (PBS, pH 7.4) (1 mL) containing 50 µL of Tween-80 (W₁ phase). In order to prepare organic phase, 100 mg of respective PB copolymers were solubilized in 4 mL of dichloromethane (DCM) with 50 µL of Span-20 (organic phase). Primary emulsion (W₁/O) was prepared by drop-wise addition of W₁ phase in organic phase under constant sonication.
Sonication was applied with probe-sonicator for 1 min at 4 W output. To avoid excessive heating and subsequent degradation of protein, the preparation of emulsion was carried out in ice-bath. Resulting W₁/O primary emulsion was then added drop-wise in 20 mL of 2% polyvinyl alcohol (PVA) solution (W₂ phase) under constant sonication for 4 min at 5 W output. Double emulsion (W₁/O/W₂) was stirred at room temperature for 30 min followed by evaporation of DCM under low pressure. Once the DCM was evaporated, NPs were centrifuged for 30 min at 20000 rpm and 4 °C followed by two washing cycles with distilled deionized water (DDW). Finally, IgG-loaded NPs were freeze-dried in presence of 5% mannitol (cryoprotectant) and stored at -20 °C until further use. NPs were prepared with two PB copolymers i.e., PB-A and PB-B. The similar protocol was utilized to prepare FITC-BSA and bevacizumab-loaded NPs. Freeze-dried NPs were evaluated for particle size, EE (%), DL (%) and in vitro drug release behavior.

Characterization of NPs

Particle size and polydispersity

Freeze-dried NPs were dispersed in DDW (1 mg/mL) and analyzed for their size and its distribution. Particle size was evaluated by particle size analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd, Worcestershire, UK) at 90° scattering angle. All the NP samples were analyzed in triplicate.

Entrapment efficiency (EE) and drug loading (DL)

Protein-encapsulated freeze-dried NPs were evaluated for the estimation of EE and DL. EE of the NPs was estimated by quantifying the amount of protein in the supernatants which were obtained during the NP preparation. Micro BCA™ protein estimation kit was employed for the quantification of total protein. In order to evaluated DL, 2 mg equivalent protein-loaded
NPs were dissolved in 200 µL of dimethyl sulfoxide (DMSO). Resulting solutions were analyzed by UV absorbance spectroscopy. Standard curve of respective proteins (IgG, FITC-BSA and bevacizumab) ranging from 31.25 to 2000 µg/mL were prepared in DMSO. Following equations were utilized for the calculation of EE (%) and DL (%).

\[
EE (\%) = \left(1 - \frac{\text{Amount of drug in supernatant}}{\text{Total amount of drug}}\right) \times 100 \quad \ldots \text{Eq. 5.3}
\]

\[
DL (\%) = \left(\frac{\text{Amount of drug in nanoparticles}}{\text{Total amount of drug and polymer}}\right) \times 100 \quad \ldots \text{Eq. 5.4}
\]

*In vitro release studies*

IgG-loaded NPs were further characterized for their ability to sustain drug release. In order to perform *in vitro* drug release studies, 1 mg of IgG equivalent freeze-dried NPs were suspended in 1 mL of PBS (pH 7.4). Resulting NPs suspension was then incubated in water bath equilibrated at 37 °C. At predefined time intervals, NPs were centrifuged at 13000 rpm for 30 min. A 200 µL of supernatant was collected and replaced with the same volume of PBS. NPs were then resuspended and release study was continued at 37 °C. In a second set of *in vitro* release studies, 1 mg drug equivalent IgG-NPs were suspended in 500 µL of aqueous solution of thermosensitive gelling polymer (PB-C) (20 wt%). Resulting suspension was incubated in 10 mL vial at 37 °C for 30 min. Once gel was solidified, 5 mL of PBS (pre-incubated at 37 °C) was slowly added. At predetermined time intervals, 1 mL of clear supernatant was collected and replaced with same volume of fresh PBS (preincubated at 37 °C). Release samples were analyzed by Micro BCA™ for total protein content. Micro BCA™ was performed according to supplier’s instructions. FITC-BSA and bevacizumab-loaded NPs were also evaluated for their *in vitro* release behavior. Release samples of FITC-BSA were
analyzed by fluorescence spectroscopy, where excitation and emission wavelengths were 490 nm and 525 nm, respectively. *In vitro* release experiments were performed in triplicates and expressed as cumulative drug released (%) with time.

**Release kinetics**

In order to investigate release mechanisms, release data were fitted to various kinetic models described below,

**Korsmeyer-Peppas equation**

\[
\frac{M_t}{M_\infty} = k t^n \quad \text{...Eq. 5.5}
\]

k is the kinetic constant and n is the diffusion exponent describes release mechanism. \(M_t\) and \(M_\infty\) represent the cumulative protein release at time t and at the equilibrium, respectively.

**Higuchi equation**

\[
Q_t = K t^{1/2} \quad \text{...Eq. 5.6}
\]

K denotes the Higuchi rate kinetic constant, \(Q_t\) is the amount of released protein at time t, and t is time in hours.

**Hixon-Crowell equation**

\[
C_0^{1/3} - C_t^{1/3} = kt \quad \text{...Eq. 5.7}
\]

\(C_0\) and \(C_t\) represents the initial amount and remaining amount of protein in formulation, respectively. k is the constant incorporating surface-volume relation and t is time in hours.

**First-order equation**

\[
\log C = \log C_0 - \frac{Kt}{2.303} \quad \text{...Eq. 5.8}
\]
K denotes the first-order rate constant, $C_0$ is the initial protein concentration and $t$ represents time in hours.

*Zero-order equation*

$$C = K_0 t$$ …Eq. 5.9

$K_0$ is the zero-order rate constant and $t$ is time in hours.

*Stability estimation of IgG*

Released IgG was analyzed by CD spectroscopy for the estimation of secondary structure. CD analysis was carried at room temperature with Jasco 720 spectropolarimeter. CD spectra were recorded between the wavelengths of 200 to 250 nm at scanning speed of 5 nm/min utilizing 1 cm cell. CD measurements were reported as molar ellipticity [θ]. CD spectrum of PBS was utilized as blank.

*Stability estimation of bevacizumab by in vitro biological assays*

*Cell proliferation assay*

A cell proliferation assay was performed according previously published protocols with minor modifications [213-215]. Concentrations of live cells were quantified by MTS assay, which was performed according to manufacturer’s instructions. Briefly, cells were seeded at the density of $5 \times 10^3$ cells/well of 96-well cell culture plate. After 24 h of incubation, cells were serum-starved overnight followed by addition of serum free medium containing 100 ng/mL of VEGF and 0.25 mg/mL of released bevacizumab (test samples) or 0.25 mg/mL of native bevacizumab. Cells without VEGF or bevacizumab were considered as negative control or cells exposed only to VEGF (100 ng/mL) as positive control. Cells were further incubated for 24 h at 37 °C and 5% CO$_2$. After completion of treatment, cells were exposed to 100 μL of
serum free medium containing 20 µL of MTS solution and incubated for 4 h. The absorbance was recorded at 450 nm using a microplate reader.

Cell migration assay

A cell migration assay was performed as described elsewhere with few modifications [216]. RF/6A cells were starved overnight (by exposing to serum free medium), trypsinized and suspended in serum free medium containing 0.25 mg/mL of bevacizumab (native or released from NPs). The 5 x 10³ cells were seeded in upper chamber of Transwell (8.0 μm pore size, 10 mm diameter; Corning Inc.), pre-incubated with cell culture medium. VEGF (100 ng/mL) was placed into the lower chamber and the cells were incubated for 24 h at 37 °C and 5% CO₂. Non-migrated cells were removed from upper chamber by cotton swab and concentration of migrated cells was estimated by AlamarBlue® assay. AlamarBlue® assay was performed according to supplier’s protocol. Moreover, for visual evidence, migrated cells were also stained with methylene blue and images were taken with Leica DMI3000B inverted microscope (Germany). Each experiment was repeated three times.

Results and discussion

Synthesis and characterization of PB copolymers

PB copolymers (PB-A and PB-B) were successfully synthesized by ring-opening bulk copolymerization of ε-caprolactone, and L-lactide/glycolide. Firstly, TB copolymers (PCL-PEG-PCL) were synthesized, purified and characterized. Purified TB copolymers were then utilized for the synthesis of respective PB copolymers i.e., PB-A (PGA-PCL-PEG-PCL-PGA) and PB-B (PLA-PCL-PEG-PCL-PLA). Purity and molecular weights (Mn) of PB copolymers were calculated by ¹H-NMR spectroscopy. As described in Figures 5.4 and 5.5, typical ¹H-NMR signals of PCL blocks were observed at 1.40, 1.65, 2.30 and 4.06 ppm depicting
methylene protons of -(CH$_2$)$_3$-, -OCO-CH$_2$-, and -CH$_2$OOC-, respectively. PB-A exhibited cluster of singlets between 4.6 to 4.9 ppm representing methylene protons (-CH$_2$-) of PGA units. PB copolymer with PLA units as terminals (PB-B) demonstrated two additional peaks at 1.50 (-CH$_3$) and 5.17 (-CH-) ppm. Molar ratios of PB-A and PB-B were calculated from the integration values of PEG (3.65 ppm), PCL (2.30 ppm), and PLA (5.17 ppm) or PGA (4.6-4.9 ppm). $^1$H-NMR spectra of PB-C depicted in (Figure 5.6) demonstrated typical proton signals of PEG, PCL and PLA. An additional peak at 3.38 ppm was denoted to terminal methyl of (-OCH$_3$-) of PEG, which was utilized for the molecular weight (Mn) calculation of PB-C copolymer. As depicted in Table 5.1, observed molecular weights (Mn) of PB-A, PB-B and PB-C were very similar to theoretical molecular weights. For the ease, in the following text, theoretical molecular weights are mentioned instead of calculated molecular weights (Mn).

**In vitro cytotoxicity studies**

In order to investigate compatibility of PB polymeric materials with biological system (ocular cell lines), 10 mg/mL concentration of PB-A and PB-B were exposed to ARPE-19, SIRC, HCEC and RAW-264.7 cells for 48 h. LDH is a cytoplasmic enzyme, secreted in cell culture medium following cell-membrane damage. Estimation of the concentration of LDH in the cell supernatant provides a direct estimation of PB copolymer toxicity. Less than 10% of LDH release was observed after 48 h of exposure period indicating negligible toxicity for any of the ocular cell lines (Figure 5.7). Noticeably, results were comparable with the respective negative controls.
Figure 5.4: $^1$H-NMR spectrum of PB-A copolymer in CDCl$_3$. 
Figure 5.5: $^1$H-NMR spectrum of PB-B copolymer in CDCl$_3$. 
Figure 5.6: $^1$H-NMR spectrum of PB-C copolymer in CDCl$_3$. 
Table 5.1: Characterization of PB copolymers.

<table>
<thead>
<tr>
<th>Code</th>
<th>Structure</th>
<th>PGA/PCL/PEG or PLA/PCL/PEG</th>
<th>Total ( Mn^a ) (theoretical)</th>
<th>Total ( Mn^b ) (calculated)</th>
<th>Total ( Mn^c ) (calculated)</th>
<th>( M_w^c ) (GPC)</th>
<th>PDI (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB-A</td>
<td>PGA(<em>{300})-PCL(</em>{750})-PEG(<em>{1000})-PCL(</em>{750})-PGA(_{300})</td>
<td>0.6/15/1</td>
<td>16600</td>
<td>15710</td>
<td>13780</td>
<td>18640</td>
<td>1.35</td>
</tr>
<tr>
<td>PB-B</td>
<td>PLA(<em>{3450})-PCL(</em>{5700})-PEG(<em>{4000})-PCL(</em>{5700})-PLA(_{3450})</td>
<td>1.73/2.85/1</td>
<td>22300</td>
<td>21030</td>
<td>19160</td>
<td>26980</td>
<td>1.41</td>
</tr>
<tr>
<td>PB-C</td>
<td>PEG(<em>{550})-PCL(</em>{825})-PLA(<em>{550})-PCL(</em>{825})-PEG(_{550})</td>
<td>0.5/1.5/1</td>
<td>3300</td>
<td>2910</td>
<td>4230</td>
<td>6040</td>
<td>1.43</td>
</tr>
</tbody>
</table>

\( a. \) Theoretical value, calculated according to the feed ratio
\( b. \) Calculated from \(^1\)H-NMR results
\( c. \) Determined by GPC analysis
Figure 5.7: *In vitro* cytotoxicity assay (LDH) of PB-A and PB-B copolymers at the concentration of 10 mg/mL was performed on ARPE-19, SIRC, HCEC and RAW-264.7 cell lines.
Figure 5.8: *In vitro* cell viability assay (MTS) of PB-A and PB-B copolymers at the concentration of 10 mg/mL was performed on ARPE-19, SIRC, HCEC and RAW-264.7 cell lines.
Figure 5.9: *In vitro* biocompatibility of PB-A and PB-B copolymers was evaluated by estimating the levels of TNF-α, IL-6 and IL-1β in the supernatants of polymer treated RAW 264.7 cells.
To further confirm the results observed for LDH assays, MTS cell viability studies were performed utilizing the similar protocol. In MTS assay, only metabolically active cells convert tetrazolium compound to formazan. Hence, the concentrations of formazan products provide a direct estimation of cell viability. Results described in Figure 5.8 demonstrated more than 90% cell viability (for all the cell lines) after 48 h exposure to polymer materials suggesting excellent safety profile of block copolymers for the ocular applications. No significant difference in cell viability was observed relative to negative control.

**In vitro biocompatibility studies**

Many investigators have utilized *in vitro* cell culture model (RAW-264.7) for the estimation of biocompatibility of polymeric materials intended for human applications. In this experiment we have estimated concentration of various cytokines such as TNF-\(\alpha\), IL-6 and IL-1\(\beta\) secreted in cell supernatant following 24 h exposure to PB-A and PB-B copolymers. Samples were analyzed via sandwich ELISA method provided by manufacturer. Results depicted in Figure 5.9 indicate significant release of TNF-\(\alpha\) (\(\sim\)200 pg/mL) in both groups i.e., PB-A and PB-B. However, these values are comparable to negative control (cells without treatment) with no significant difference. Similarly, negligible release of IL-6 and IL-1\(\beta\) were observed suggesting outstanding safety profile of PB copolymers.

**Characterization of NPs**

*Particle size and polydispersity*

IgG, FITC-BSA and bevacizumab-encapsulated PB NPs were prepared by \(W_1/O/W_2\) double emulsion solvent evaporation method. NPs prepared from PB copolymers were ranging from 320-355 nm (Table 5.2). Moreover, we have observed unimodal size distribution with very narrow polydispersity (0.273-0.305). We did not observe any significant effect of polymer
composition (PB-A/PB-B) or type of protein molecule (IgG/BSA/bevacizumab) on particle size. These results suggest that hydrodynamic diameter of protein therapeutics or polymer composition has little or no effect on particle size or its distribution.

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

EE and DL are significantly influenced by various parameters including copolymer composition (hydrophobicity of polymer) and volumes of various phases (W₁, O and W₂). In order to understand the effect of hydrophobicity of copolymers on EE and DL, we have prepared and evaluated IgG and FITC-BSA-loaded NPs with PB-A and PB-B. It is important to note that PB-A copolymer comprising PGA (hydrophilic block) is relatively hydrophilic copolymer than PB-B copolymer comprising PLA (hydrophobic block). As described in Table 5.2, encapsulation of IgG or FITC-BSA in PB-A NPs were ~40% and ~35%, respectively. However, PB-B NPs exhibited significantly higher encapsulation for IgG (~70%) and FITC-BSA (~69%) relative to PB-A NPs. This may attributed to the fact that during the preparation of NPs (solvent evaporation), high hydrophobicity may allow faster precipitation of PB-B copolymer to form NPs preventing diffusion of IgG/FITC-BSA from W₁ phase to external aqueous (W₂) phase. This phenomenon possibly ensured higher EE of protein therapeutics in PB-B NPs. Due to higher hydrophilicity, PB-A copolymer may remain hydrated with W₁ and W₂ phase (during NPs preparation) allowing escape of IgG/FITC-BSA in external phase resulting poor EE. However, we did not observe any effect of hydrodynamic diameter of IgG or FITC-BSA on EE or DL. It may possible that both the proteins are too large (≥ 66 kDa) to show any significant effect of hydrodynamic diameter on EE or DL. Bevacizumab-loaded PB-B NPs exhibited ~67% of EE and ~6% of DL, very similar to IgG-loaded PB-B NPs (Table 5.2) suggested that bevacizumab may behaved very similar to IgG during NP preparation.
In vitro release studies

In order to evaluate the effect of polymer hydrophobicity, release of FITC-BSA and IgG from PB-A and PB-B NPs were evaluated. As described in Figure 5.10, both NPs (PB-A and PB-B) demonstrated biphasic release profile i.e., initial burst release followed by sustained release. PB-A NPs exhibited significantly higher burst release (~56%) of FITC-BSA relative to PB-B NPs (~48%). In a second phase of release, PB-B NPs sustained release of BSA for ~36 days whereas PB-A NPs prolonged the release for only ~27 days. Similarly, effects of polymer hydrophobicity were observed with IgG-encapsulated NPs where PB-B NPs displayed prolonged release (~44 days) than PB-A NPs (~30 days) (Figure 5.11). As described earlier, PGA based PB-A copolymer is hydrophilic than the PLA based PB-B copolymer. Therefore, it is anticipated that PB-A NPs possibly have higher affinity for the protein molecules which may allow higher amount of surface adsorbed drug. Moreover, being hydrophilic, PB-A NPs may get easily hydrated and allow easy diffusion of water molecules through the polymer matrix. Both, higher amount of surface adsorbed proteins and higher affinity towards water molecules may have simultaneously contributed to the higher burst release and shorter duration of release period.

We have hypothesized that hydrodynamic diameter of protein therapeutics has significant effect on drug release pattern. In order to confirm that we have compared in vitro release profiles of FITC-BSA (66 kDa) and IgG (150 kDa) (Figure 5.12) from PB-B NPs. Results clearly indicated significantly higher burst release and shorter release duration for FITC-BSA relative to IgG from their respective NPs. It may be attributed to the fact that FITC-BSA has smaller hydrodynamic diameter compared to IgG which may lead to faster diffusion through the polymer matrix of NPs. To further confirm this hypothesis, we have compared in
*vitro* release profile of IgG and bevacizumab (149 kDa) from their respective PB-B NPs. Results reported in Figure 5.13 described no significant difference between the release profile of IgG and bevacizumab. This may be due to the fact that IgG and bevacizumab are full length antibodies with similar hydrophilicity and molecular weight (hydrodynamic diameter). Hence, both protein molecules might behave alike during NPs preparation and also during release study. These results indicate that there is a little or no effect of the type of protein (IgG or bevacizumab) on drug release pattern but there is a significant effect of hydrodynamic diameter (FITC-BSA or IgG).

Protein therapeutics possess very high specificity hence require very low dose to assert their therapeutic action. Any nanoparticulate or microparticulate system have certain amount of surface adsorbed drug, which releases within first 24 h giving burst effect. Being very potent by nature, burst release of protein therapeutics may produce serious side effects. Therefore, pharmaceutical scientists are focused to develop a formulation which can eliminate burst effect and offer zero-order drug release throughout a release period. In order to achieve zero-order drug release profile, we have suspended protein-encapsulated PB-B NPs in an aqueous solution of thermosensitive gelling polymer (PB-C). Thermosensitive gelling solution was composed of 20 wt% PB-C copolymer in DDW. Aqueous solution of PB-C copolymer remains liquid at room temperature or below and immediately transforms to hydrogel at body temperature (sol-gel transition curve is not shown).
Table 5.2: Characterization of FITC-BSA and IgG-loaded NPs.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Polymers</th>
<th>Entrapment efficiency (%)</th>
<th>Loading (%)</th>
<th>Particle size (nm)</th>
<th>Polydispersity</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC-BSA</td>
<td>PB-A</td>
<td>35.0 ± 3.7</td>
<td>5.0 ± 0.4</td>
<td>350 ± 30</td>
<td>0.286</td>
</tr>
<tr>
<td></td>
<td>PB-B</td>
<td>69.5 ± 6.2</td>
<td>5.4 ± 0.5</td>
<td>320 ± 20</td>
<td>0.280</td>
</tr>
<tr>
<td>IgG</td>
<td>PB-A</td>
<td>40.5 ± 3.5</td>
<td>6.3 ± 0.3</td>
<td>370 ± 10</td>
<td>0.305</td>
</tr>
<tr>
<td></td>
<td>PB-B</td>
<td>70.1 ± 4.1</td>
<td>6.1 ± 0.3</td>
<td>350 ± 10</td>
<td>0.273</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>PB-B</td>
<td>67.3 ± 3.3</td>
<td>6.1 ± 0.2</td>
<td>340 ± 20</td>
<td>0.246</td>
</tr>
</tbody>
</table>
Figure 5.10: *In vitro* release of FITC-BSA from NPs prepared with PB-A and PB-B copolymers.
**Figure 5.11:** *In vitro* release of IgG from NPs prepared with PB-A and PB-B copolymers.
Figure 5.12: *In vitro* release of FITC-BSA and IgG from PB-B NPs
Figure 5.13: *In vitro* release of IgG and bevacizumab from PB-B NPs
Figure 5.14: *In vitro* release of FITC-BSA from PB-B NPs and PB-B NPs suspended in PB-C gelling polymer
Figure 5.15: *In vitro* release of IgG from PB-B NPs and PB-B NPs suspended in PB-C gelling polymer (20 wt%).
Protein-loaded NPs were suspended in 20 wt% gelling solution and exposed to 37°C which immediately transformed to solid hydrogel entrapping NPs throughout the polymer matrix. These composite formulations comprised of protein-loaded PB-B NPs (FITC-BSA and IgG) suspended in thermosensitive gel were evaluated for their release behavior. As described in Figure 5.14, burst release of FITC-BSA from composite formulation was negligible (~10%) relative to burst release observed from PB-B NPs (~48%) alone. In addition, release of FITC-BSA was prolonged for ~40 days. The similar behavior was observed for a composite formulation comprising IgG-loaded PB-B NPs suspended in 20 wt% thermosensitive gelling solution (Figure 5.15). Composite formulation of IgG exhibited negligible burst release followed zero-order release up to ~60 days. This behavior may be due to the fact that when the NPs were suspended into the gel matrix, gel matrix might serve as an additional diffusion barrier for the surface adsorbed drug. The gel matrix might deter the dumping of surface adsorbed dose eliminating burst effect which eventually provided nearly zero-order drug release throughout the release period.

**Release kinetics**

In order to evaluate drug release mechanism, we have fitted *in vitro* drug release data in five different release kinetic models i.e., Korsmeyer-Peppas, Higuchi, Hixon-Crowell, zero-order and first-order. Results showed in Table 5.3, indicated that Korsmeyer-Peppas was the best fit model for all the formulations with $R^2$ values ranging between 0.977-0.997. Moreover, $n$ values in Korsmeyer-Peppas model for release of FITC-BSA, IgG and bevacizumab from PB-B NPs were below 0.43 indicating diffusion controlled release. Interestingly, $n$ values for composite formulation of FITC-BSA (0.549) and IgG (0.818) (NPs suspended in thermosensitive gel) were between 0.43-0.89 suggesting anomalous diffusion. In other words,
release of protein therapeutics from composite formulation is controlled by diffusion as well as degradation of polymer.

**Stability of secondary structure of IgG confirmed by CD spectroscopy**

It is obvious that protein therapeutics need to maintain their three dimensional structure to exert pharmacological action. CD spectroscopy is a sensitive and robust analytical technique exploited for the investigation of secondary and at some extent tertiary conformation of proteins. It is sensitive enough to detect minor conformational changes in α-helix and β-sheets of the protein. Therefore, we have utilized this technique to confirm the conformational stability of released IgG and compared with CD spectrum of native IgG (Figure 5.16). CD spectrum of released IgG demonstrated λ minima of 218 nm similar to the native IgG. In addition, the CD spectra of native IgG and released IgG ranging from 200 nm to 250 nm were identical to each other indicating retention of structural conformation during NP preparation.

**Cell proliferation assay**

A cell proliferation assay was performed as described previously [215]. RF/6A cells proliferated rapidly in presence of VEGF (100 ng/mL, +ve control) whereas its proliferation was inhibited in absence of VEGF indicating sensitivity of endothelial cells toward growth factor particularly, VEGF (Figure 5.17). Native bevacizumab (standard group) has strongly inhibited the VEGF-induced cell proliferation at the concentration of 0.25 mg/mL. The similar level of inhibition was observed when released (released from NPs) bevacizumab (test/sample group) was exposed for 24h to VEGF treated cells. Moreover, inhibitory effects of released and native bevacizumab were not significantly different than -ve control.
**Cell migration assay**

Chemo-attractant property of VEGF stimulates RF/6A cell migration of across a porous membrane toward a VEGF stimulus. Biological activity of released bevacizumab was further evaluated by VEGF-induced cell migration assay. As described in Figure 5.18, a test/sample group (released bevacizumab) exhibited significant inhibition of cell migration across the transwell membrane relative to VEGF group (+ve control). Results showed that this inhibitory effect was not significantly different than -ve control or a standard group (cells treated with native bevacizumab). For the visual evidence, migrated cells were stained with methylene blue and images were taken. Data depicted in Figure 5.18b also supported the results described earlier.

Data observed in cell proliferation and cell migration assays clearly pointed out that bevacizumab has retained its biological activity during the process of NP preparation. Previous reports suggest that PLA and/or PGA based copolymer produce large molar mass of lactic acid and/or glycolic acid [183, 197]. These degradation products stimulate hydrolytic degradation of protein therapeutics. Retention of protein stability (IgG and bevacizumab) in PB NPs may attributed to lower molar mass of PLA or PGA blocks which produce very low amounts of lactic acid or glycolic acid, eliminating or reducing protein degradation.
Table 5.3: Coefficient of determination ($R^2$) for various kinetic models for \textit{in vitro} release of FITC-BSA, IgG and bevacizumab.

<table>
<thead>
<tr>
<th>Block copolymers</th>
<th>Korsmeyer-Peppas</th>
<th>Higuchi</th>
<th>Hixson-Crowell</th>
<th>First-Order</th>
<th>Zero-Order</th>
<th>Best fit model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R^2$</td>
<td>n</td>
<td>$R^2$</td>
<td>$R^2$</td>
<td>$R^2$</td>
<td></td>
</tr>
<tr>
<td>FITC-BSA PB-B NPs</td>
<td>0.989</td>
<td>0.327</td>
<td>0.945</td>
<td>0.905</td>
<td>0.984</td>
<td>0.783</td>
</tr>
<tr>
<td>IgG NPs PB-B NPs</td>
<td>0.982</td>
<td>0.334</td>
<td>0.979</td>
<td>0.979</td>
<td>0.959</td>
<td>0.898</td>
</tr>
<tr>
<td>Bevacizumab PB-B NPs</td>
<td>0.977</td>
<td>0.344</td>
<td>0.969</td>
<td>0.961</td>
<td>0.959</td>
<td>0.881</td>
</tr>
<tr>
<td>FITC-BSA PB-B NPs suspended in gel</td>
<td>0.992</td>
<td>0.549</td>
<td>0.978</td>
<td>0.959</td>
<td>0.982</td>
<td>0.851</td>
</tr>
<tr>
<td>IgG PB-B NPs suspended in gel</td>
<td>0.997</td>
<td>0.818</td>
<td>0.971</td>
<td>0.996</td>
<td>0.987</td>
<td>0.994</td>
</tr>
</tbody>
</table>
Figure 5.16: Stability of released IgG confirmed by CD spectroscopy.
Figure 5.17: Cell proliferation assay performed on RF/6A cells to evaluate the biological activity of bevacizumab (a) absorbance produced by live cells (b) images of live cells where (1) negative control (untreated cells), (2) standard (cells exposed to 100 ng/mL of VEGF and 0.25 mg/mL of bevacizumab), (3) sample (cells exposed to 100 ng/mL of VEGF and 0.25 mg/mL of released bevacizumab), and (4) positive control (cell exposed to 100 ng/mL VEGF).
Figure 5.18: Cell migration assay performed on RF/6A cells to evaluate the biological activity of bevacizumab (a) fluorescence intensity indicating number of migrated cells (b) images of migrated cells stained by methylene blue where (1) negative control (untreated cells), (2) standard (cells exposed to 100 ng/mL of VEGF and 0.25 mg/mL of bevacizumab), (3) sample (cells exposed to 100 ng/mL of VEGF and 0.25 mg/mL of released bevacizumab), and (4) positive control (cell exposed to 100 ng/mL VEGF).
Conclusion

We have successfully synthesized and characterized novel PB copolymers for the preparation of NPs and thermosensitive gel. In order to eliminate burst release phase, a novel composite formulation comprised of protein-loaded PB NPs suspended in a PB thermosensitive gel was successfully formulated and evaluated. FITC-BSA and IgG-encapsulated NPs suspended in thermosensitive gel demonstrated continuous zero-order release, avoiding the possibility of dose dependent toxicity that may occur due to an initial burst release. Moreover, CD spectroscopy demonstrated the retention of structural conformation of released IgG. Bevacizumab-loaded NPs demonstrated similar patterns of burst release and sustained release as of IgG-embedded NPs. *In vitro* cell proliferation and cell migration assay confirmed retention of biological activity of the bevacizumab. This approach can act as a platform for the ocular delivery of therapeutic macromolecules, and can minimize the side effects associated with frequent intravitreal injections.
CHAPTER 6

OPTIMIZATION OF NOVEL PENTABLOCK COPOLYMER BASED COMPOSITE FORMULATION FOR SUSTAINED DELIVERY OF PROTEIN THERAPEUTICS IN THE TREATMENT OF OCULAR DISEASES

Rationale

The ideal formulation for the intravitreal delivery should possess following characteristics, (a) high drug loading in small volume (≤ 100 µL) that lasts up to 6 months or more, (b) provide constant release (zero-order release) throughout the release period without any burst effect, (c) easy to administered such as injectable system and not implants, (d) ensure stability of protein/peptide, (e) biodegradable and biocompatible, and (f) the time required for biodegradation of formulation should not be more than 1.5 times the release period.

Many researchers are investigating protein-encapsulated NPs for the sustained delivery in the treatment of back of the eye diseases. It is easy to achieve high drug loading in NPs for hydrophobic therapeutic agents. In contrast, it is very difficult to achieve high drug loading of protein therapeutics (hydrophilic agents). One more limitation for intravitreal injection is small injection volume (≤100 µL). Therefore, one of the important challenges is to develop a formulation which can carry large amount of dose in limited injection volume and provide constant release up to 6 months or more.

In order to address mentioned issues, we have synthesized novel biodegradable and biocompatible PB copolymers composed of PEG, PCL, and PLA/PGA. In this section, we study the effects of various formulation parameters on EE, DL and in vitro release profile. In order to incorporate a large amount of protein therapeutic in small volume of formulation, we have optimized NP preparation methods to attain maximum possible DL. To achieve constant
(zero-order) release, we have utilized a novel concept of composite formulation (discussed in chapter 5) where protein/peptide-loaded PB NPs were suspended in a PB thermosensitive gel. Moreover, the optimized methods for NP preparation were also applied to encapsulate proteins or peptides with different molecular weights ranging from 1-237 kDa. Biological activity of released proteins or peptides was also determined utilizing enzymatic activity assays.

**Materials and methods**

**Materials**

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-Aldrich (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™ and catalase colorimetric assay kits 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, PLA-PCL-PEG-PCL-PLA (PB-A/PB-B), PGA-PCL-PEG-PCL-PGA (PB-C/PB-D), and PEG-PCL-PLA-PCL-PEG (PB-E) were synthesized by ring-opening bulk copolymerization [217]. 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. TB (TB) copolymer (PCL-
PEG-PCL) was purified by dissolving 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. Reaction for the synthesis of PB-A/PB-B were carried out at 130 °C for 24 h whereas for PB-C/PB-D at 200 °C for 24 h. At the end, 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 synthesized, purified and characterized according to previously published protocol with minor modifications [217]. For the synthesis of PB-E, TB copolymer (mPEG-PCL-PLA) was 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. Coupling reaction was carried out for 8 h at 70 °C. 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 6.1 and 6.2.
Figure 6.1a: Synthesis scheme for PB-A and PB-B (PLA-PCL-PEG-PCL-PLA)

Step 1

\[
\begin{align*}
&\text{PEG} \quad \text{\(\varepsilon\) caprolactone} \\
&\text{OH}[\begin{array}{c}
\text{H}_2 \quad \text{H}_2 \\
\text{CH}_2 \quad \text{CH}_2 \\
\end{array}]_x + \overset{\text{Sn(Oct)\textsubscript{2}}}{{}\frac{130^\circ\text{C}}{}{}}} \rightarrow \text{HO-PCL-PEG-PCL-OH} \\
&\text{HO}[\begin{array}{c}
\text{H}_2 \quad \text{C} \\
\text{H}_2 \quad \text{C} \\
\end{array}]_x \quad \overset{\text{Sn(Oct)\textsubscript{2}}}{{}\frac{130^\circ\text{C}}{}{}}} \rightarrow \text{HO-PCL-PEG-PCL-OH} \\
&\text{HO}[\begin{array}{c}
\text{H}_2 \quad \text{C} \\
\text{H}_2 \quad \text{C} \\
\end{array}]_y \quad \overset{\text{Sn(Oct)\textsubscript{2}}}{{}\frac{200^\circ\text{C}}{}{}}} \rightarrow \text{PGA-PCL-PEG-PCL-PGA}
\end{align*}
\]

Figure 6.1b: Synthesis scheme for PB-C and PB-D (PGA-PCL-PEG-PCL-PGA).
**Figure 6.2:** Synthesis scheme for PB-E (PEG-PCL-PLA-PCL-PEG).
Characterization of polymers

PB copolymers were characterized for their molecular weight and purity by \(^1\)H-NMR spectroscopy and gel permeation chromatography (GPC).

\(^1\)H-NMR

Polymeric materials (5 mg) were dissolved in 600 µL of CDCl\(_3\) and were analyzed on Varian-400 MHz NMR instrument. Molecular weight (Mn) and purity of the polymers were evaluated from the \(^1\)H-NMR spectrum.

GPC

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

Preparation of thermosensitive gelling solution

To prepare 20 wt% aqueous gelling 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 aqueous solution was confirmed by vial inverting method reported earlier [179]. Aqueous solution 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 NPs

Protein/peptide-loaded PB-NPs were prepared by W\(_1\)/O/W\(_2\) double emulsion solvent evaporation method [201]. Briefly, a peptide or protein aqueous solution (W\(_1\) 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 organic phase was then evaporated under vacuum using rotavap to obtain NPs. NPs were separated by ultracentrifugation at 20,000 rpm for 30 min (4 °C). Particles were washed twice with distilled deionized water (DDW), and centrifuged to remove traces of PVA and unentrapped 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 behavior. Process parameters such as phase volume ratio, drug/polymer ratio, and types of polymer were optimized to achieve higher DL. The detailed process parameters are reported in Table 6.2.

**Characterization of NPs**

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

Protein/peptide-encapsulated freeze-dried NPs were evaluated for the estimation of EE and DL. Amount of unentrapped protein/peptide in supernatant was determined by micro BCA™ protein assay kit following manufacturer’s protocol. Standard curve of respective proteins/peptides (octreotide, insulin, lysozyme, IgG-Fab, IgG and catalase) were prepared in the range of 3-200 µg/mL. Following equations were utilized for the calculation of EE (%) and DL (%).

EE (%) was calculated with eq. 6.1

\[
EE \, (\%) = \left(1 - \frac{\text{Amount of drug in supernatant}}{\text{Total amount of drug}}\right) \times 100 \quad \ldots \text{Eq. 6.1}
\]
DL (%) was calculated by eq. 6.2

\[
DL (%) = \left( \frac{\text{Amount of drug in nanoparticles}}{\text{Total amount of drug and polymer}} \right) \times 100 \quad \ldots \text{Eq. 6.2}
\]

**In vitro release studies**

Protein/peptide-loaded freeze-dried NPs were characterized for their release behavior. To perform *in vitro* drug release from composite formulation, precalculated amount of NPs were suspended in 100 µL of PB-E thermosensitive gelling solution (20 wt%) maintained at 4 °C. Resulting 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). Release samples were evaluated for protein content by Micro BCA™ total protein assay which was performed according to supplier’s instructions. Release experiments were carried out in triplicates and depicted as cumulative drug release (%) against time. Biological activity of lysozyme and catalase were confirmed by their enzymatic assays.

**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 (\(\log C = \log C_0 - Kt/2.303\)), and Zero-order (\(C = K_0 t\)). Based on the R² value, best fit model was identified. Diffusion exponent (n) of Korsmeyer-Peppas equation was utilized to understand the mechanism of release.
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 controlled samples. Controls were comprised of lysozyme solution incubated at 37 °C in PBS (pH 7.4) which kept 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. A 100 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 [218].

\[
\text{Units of lysozyme in one mL sample} = \frac{(\Delta A_{450 \text{ nm/min Test}} - \Delta A_{450 \text{ nm/min Blank})(df)}}{(0.001)(0.1)} \quad \text{…Eq. 6.3}
\]

\[
\frac{\text{Units of lysozyme \ mg of sample}}{\text{mg of sample}} = \frac{\text{Units of lysozyme/mL of sample}}{\text{mg of lysozyme/mL of sample}} \quad \text{…Eq. 6.4}
\]

Where, 0.001 was obtained from the definition of lysozyme as one unit of enzyme is able to produce \(\Delta\text{Abs}_{450\text{nm}}\) of 0.001 per minute at pH 6.15 and 25 °C utilizing Micrococcus luteus suspension, 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 of the same time points.
Estimation of enzymatic activity of catalase

Enzymatic activity of catalase was estimated for in vitro release samples. Control samples with known concentration of catalase were prepared in PBS (pH 7.4) and exposed at 37 °C along with in vitro release samples. Control, test and standard samples were analyzed for catalase activity with catalase colorimetric assay kit. Assay was performed according to supplier’s protocol. Briefly, standards with known concentrations were prepared in assay buffer. A 25 µL of standard, control or sample was added in 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. Plate was incubated for 15 min at room temperature and then analyzed by UV spectrophotometer at 570 nm. According to catalase activity assay, 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. Firstly, TB copolymers (PCL-PEG-PCL or mPEG-PCL-PLA) were synthesized, purified and characterized. Resulting TB copolymers were utilized for the preparation of PB copolymers i.e., PB-A/PB-B (PLA-PCL-PEG-PCL-PLA), PB-C/PB-D (PGA-PCL-PEG-PCL-PGA) and PB-E (PEG-PCL-PLA-PCL-PEG). Molecular weights (Mn) and purity of the PB copolymers were examined by 1H-NMR spectroscopy. As depicted in Figures 6.3, 6.4 and 6.5, PCL blocks exhibited typical 1H-NMR peaks at 1.40, 1.65, 2.30 and 4.06 ppm attributed to methylene protons of -(CH₂)₃-.
OCO-CH₂-, and -CH₃OOC-, respectively. PB-A, PB-B and PB-E, L-lactide containing PB copolymers demonstrated two ¹H-NMR peaks at 5.17 and 1.50 ppm representing -CH- and -CH₃- groups. Similarly, PB-C and PB-D copolymers comprised of glycolic acid exhibited series of singlets between 4.6 to 4.9 ppm explaining methylene protons of PGA block. ¹H-NMR of PB-E copolymer exhibited an additional peak at 3.38 ppm which was denoted to terminal methyl of (-OCH₃-) of PEG. Molecular weight of PB copolymers were calculated from the integration values of ¹H-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 weight calculated from ¹H-NMR is reported in Table 6.1.
Figure 6.3: $^1$H-NMR spectrum of PB-A copolymer in CDCl$_3$. 
Figure 6.4: $^1$H-NMR spectrum of PB-D copolymer in CDCl$_3$. 
Figure 6.5: $^1$H-NMR spectrum of PB-E copolymer in CDCl$_3$. 
Table 6.1: Characterization of polymers.

<table>
<thead>
<tr>
<th>Code</th>
<th>Structure</th>
<th>Total Mn&lt;sup&gt;a&lt;/sup&gt; (theoretical)</th>
<th>Total Mn&lt;sup&gt;b&lt;/sup&gt; (calculated)</th>
<th>Total Mn&lt;sup&gt;c&lt;/sup&gt; (calculated)</th>
<th>M&lt;sub&gt;W&lt;/sub&gt; &lt;sup&gt;c&lt;/sup&gt; (GPC)</th>
<th>PDI&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB-A</td>
<td>PLA&lt;sub&gt;3000&lt;/sub&gt;-PCL&lt;sub&gt;7000&lt;/sub&gt;-PEG&lt;sub&gt;2000&lt;/sub&gt;-PCL&lt;sub&gt;7000&lt;/sub&gt;-PLA&lt;sub&gt;3000&lt;/sub&gt;</td>
<td>3/7/1</td>
<td>22000</td>
<td>20780</td>
<td>17250</td>
<td>24020</td>
</tr>
<tr>
<td>PB-B</td>
<td>PLA&lt;sub&gt;3000&lt;/sub&gt;-PCL&lt;sub&gt;7000&lt;/sub&gt;-PEG&lt;sub&gt;4000&lt;/sub&gt;-PCL&lt;sub&gt;7000&lt;/sub&gt;-PLA&lt;sub&gt;3000&lt;/sub&gt;</td>
<td>1.5/3.5/1</td>
<td>24000</td>
<td>21350</td>
<td>17640</td>
<td>23830</td>
</tr>
<tr>
<td>PB-C</td>
<td>PGA&lt;sub&gt;3000&lt;/sub&gt;-PCL&lt;sub&gt;7000&lt;/sub&gt;-PEG&lt;sub&gt;4000&lt;/sub&gt;-PCL&lt;sub&gt;7000&lt;/sub&gt;-PGA&lt;sub&gt;3000&lt;/sub&gt;</td>
<td>1.5/3.5/1</td>
<td>24000</td>
<td>21290</td>
<td>16180</td>
<td>23210</td>
</tr>
<tr>
<td>PB-D</td>
<td>PGA&lt;sub&gt;3000&lt;/sub&gt;-PCL&lt;sub&gt;7000&lt;/sub&gt;-PEG&lt;sub&gt;2000&lt;/sub&gt;-PCL&lt;sub&gt;7000&lt;/sub&gt;-PGA&lt;sub&gt;3000&lt;/sub&gt;</td>
<td>3/7/1</td>
<td>22000</td>
<td>20120</td>
<td>17030</td>
<td>24190</td>
</tr>
<tr>
<td>PB-E</td>
<td>PEG&lt;sub&gt;550&lt;/sub&gt;-PCL&lt;sub&gt;825&lt;/sub&gt;-PLA&lt;sub&gt;550&lt;/sub&gt;-PCL&lt;sub&gt;825&lt;/sub&gt;-PEG&lt;sub&gt;550&lt;/sub&gt;</td>
<td>0.5/1.5/1</td>
<td>3300</td>
<td>3280</td>
<td>4330</td>
<td>6100</td>
</tr>
</tbody>
</table>

a. Theoretical value, calculated according to the feed ratio
b. Calculated from <sup>1</sup>H-NMR results
c. Determined by GPC analysis
Table 6.2: Optimization of process parameters for the preparation of IgG-Fab-loaded PB NPs.

<table>
<thead>
<tr>
<th>Batch #</th>
<th>Process parameters</th>
<th>EE (%)</th>
<th>Drug loading (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Polymer types</td>
<td>Polymer (mg)</td>
<td>IgG-Fab (mg)</td>
</tr>
<tr>
<td>Batch 1</td>
<td>PB-A</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>Batch 2</td>
<td>PB-B</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>Batch 3</td>
<td>PB-A</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>Batch 4</td>
<td>PB-B</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>Batch 5</td>
<td>PB-A</td>
<td>10</td>
<td>2.5</td>
</tr>
<tr>
<td>Batch 6</td>
<td>PB-A</td>
<td>10</td>
<td>2.5</td>
</tr>
<tr>
<td>Batch 7</td>
<td>PB-A</td>
<td>10</td>
<td>2.5</td>
</tr>
<tr>
<td>Batch 8</td>
<td>PB-A</td>
<td>10</td>
<td>2.5</td>
</tr>
</tbody>
</table>

a. W₂ phase was comprised of 2% PVA
b. W₃ phase was comprised of 2% PVA
Purity, molecular weight (Mn and Mw) and polydispersity were further evaluated by GPC. As described in Table 6.1, polydispersity of all the polymers were below 1.45 suggesting narrow distribution of molecular weights. Molecular weights 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 Mn values obtained from GPC analysis were noticeably smaller than Mn values observed from $^1$H-NMR spectroscopy. This result was attributed to the difference in hydrodynamic diameter of block copolymers relative to parent homopolymers [219]. Calculated molecular weights were very similar to the theoretical molecular weights obtained from feed ratio. Hence, in the following text theoretical molecular weights are mentioned instead of calculated molecular weights.

**Preparation and characterization of NPs**

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 studied 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 type or hydrophobicity on DL where PB-A copolymer is more hydrophobic than PB-B copolymer. As represented in Table 6.2, 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 and 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. During solvent evaporation, high hydrophobicity may allow faster polymer precipitation to form NPs preventing diffusion of IgG-Fab in external aqueous (W₂) phase.

Reports published elsewhere suggested significant effect of NaCl on EE and DL in nanoparticulate system [220]. As described in Batches 3 and 4, incorporation of NaCl in W₁ (1%) and W₂ (10%) phase exhibited higher EE and loading of IgG-Fab in both PB-A (EE = ~55% and DL = 6.68%) and PB-B (EE = ~49% and DL = 5.88%) NPs 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 W₁ and W₂ phase. Higher concentration of drug in W₁ phase may enhance osmotic pressure in the internal phase which facilitates diffusion of water from external (W₂) phase. Process of diffusion may result in thinning of organic phase and eventually in lower EE and DL. Addition of salt in external phase may helped to balance osmotic pressure between W₁ and W₂ phases which resulted in lower diffusion of external phase and higher DL. However, addition of salt did not exhibit significant effect on IgG-Fab loading. This may be due to the colligative characteristic of osmotic pressure, hence, this effect may enhance the loading of lower molecular weight drug drastically relative to higher molecular weight proteins. Based on these results, PB-A copolymer was utilized for further optimization of NP preparation.

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 suggest that as drug/polymer ratio was increased from 1/10 to 1/4, EE was significantly reduced which is in accordance to the previously published results [221]. This might have resulted from 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 a ratio of 1/4 (drug/polymer). Despite the high drug/polymer ratio with Batch 5, there was no significant difference observed in DL compared to earlier PB-A NPs (Batch 1). The poor DL could be attributed to high volume of external aqueous (W\textsubscript{2}) phase. Hence, in further preparation the drug/polymer ratio was kept constant at 1/4 and effect of external phase (W\textsubscript{2}) volume was evaluated. For instance, in Batch 6 total external phase (W\textsubscript{2}) volume was lowered to 5 mL. In addition, double emulsion was prepared with only 2 mL of external aqueous (W\textsubscript{2}) phase to reduce partition of protein in water (W\textsubscript{2} phase). The resulting multiple emulsion was stabilized by dilution with 3 ml of 2% PVA (W\textsubscript{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 has significantly improved EE relative to Batch 5.

Based on results from Batch 6, it can be inferred that reduction in external phase (W\textsubscript{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\textsubscript{2} would diminish partition of protein in aqueous phase improving loading efficiency. In addition, a reduction in organic phase volume would increase polymer concentration that may lead to faster 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 of 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 Table 6.3, the size (molecular weight) of protein or peptide have significant effect on DL and EE. Interestingly, peptides or 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 difference of EE and DL were observed 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 or peptide molecules of certain hydrodynamic diameter or molecular weight behave similarly during a process of NP preparation.

W₁/O/W₂ 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 6.4, 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 6.5) was 17.19 and 16.34% (Batches 25 and 28), which was significantly higher relative to PB-C copolymer. The 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 compare to PB-C copolymer (Batches 20 and 23).
Table 6.3: Encapsulation of various proteins with optimized methods A and B (PB-A).

<table>
<thead>
<tr>
<th>Method</th>
<th>Batch #</th>
<th>Protein</th>
<th>EE (%)</th>
<th>Loading (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method A</td>
<td>Batch 9</td>
<td>Octreotide (1 kDa)</td>
<td>35.8 ± 3.6</td>
<td>8.2 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Batch 10</td>
<td>Insulin (5.8 kDa)</td>
<td>38.4 ± 0.8</td>
<td>8.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Batch 11</td>
<td>Lysozyme (14.7 kDa)</td>
<td>37.6 ± 1.7</td>
<td>8.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Batch 7</td>
<td>IgG-Fab (48 kDa)</td>
<td>69.6 ± 9.0</td>
<td>15.6 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Batch 12</td>
<td>IgG (149 kDa)</td>
<td>74.6 ± 1.3</td>
<td>15.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Batch 13</td>
<td>Catalase (237 kDa)</td>
<td>78.6 ± 0.8</td>
<td>16.4 ± 0.1</td>
</tr>
<tr>
<td>Method B</td>
<td>Batch 14</td>
<td>Octreotide (1 kDa)</td>
<td>26.9 ± 1.6</td>
<td>6.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Batch 15</td>
<td>Insulin (5.8 kDa)</td>
<td>36.4 ± 2.3</td>
<td>8.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Batch 16</td>
<td>Lysozyme (14.7 kDa)</td>
<td>38.4 ± 2.4</td>
<td>8.8 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Batch 8</td>
<td>IgG-Fab (48 kDa)</td>
<td>73.2 ± 0.2</td>
<td>15.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Batch 17</td>
<td>IgG (149 kDa)</td>
<td>54.6 ± 2.6</td>
<td>12.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Batch 18</td>
<td>Catalase (237 kDa)</td>
<td>76.9 ± 0.6</td>
<td>16.1 ± 0.1</td>
</tr>
</tbody>
</table>
Table 6.4: Encapsulation of various proteins with optimized methods A and B (PB-C).

<table>
<thead>
<tr>
<th>Method</th>
<th>Batch #</th>
<th>Protein</th>
<th>EE (%)</th>
<th>Loading (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method A</td>
<td>Batch 19</td>
<td>IgG-Fab (48 kDa)</td>
<td>46.9 ± 0.8</td>
<td>10.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Batch 20</td>
<td>IgG (149 kDa)</td>
<td>72.0 ± 0.7</td>
<td>15.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Batch 21</td>
<td>Catalase (237 kDa)</td>
<td>75.8 ± 1.2</td>
<td>15.9 ± 0.2</td>
</tr>
<tr>
<td>Method B</td>
<td>Batch 22</td>
<td>IgG-Fab (48 kDa)</td>
<td>57.2 ± 8.5</td>
<td>12.5 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Batch 23</td>
<td>IgG (149 kDa)</td>
<td>68.5 ± 1.4</td>
<td>14.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Batch 24</td>
<td>Catalase (237 kDa)</td>
<td>72.1 ± 0.7</td>
<td>15.3 ± 0.1</td>
</tr>
</tbody>
</table>
Table 6.5: Encapsulation of various proteins with optimized methods A and B (PB-D).

<table>
<thead>
<tr>
<th>Method</th>
<th>Batch #</th>
<th>Protein</th>
<th>EE (%)</th>
<th>Loading (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batch 25</td>
<td>IgG-Fab (48 kDa)</td>
<td>83.1 ± 1.4</td>
<td>17.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Batch 26</td>
<td>IgG (149 kDa)</td>
<td>85.3 ± 0.4</td>
<td>17.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Batch 27</td>
<td>Catalase (237 kDa)</td>
<td>72.9 ± 1.2</td>
<td>15.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Method B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batch 28</td>
<td>IgG-Fab (48 kDa)</td>
<td>78.1 ± 0.4</td>
<td>16.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Batch 29</td>
<td>IgG (149 kDa)</td>
<td>81.8 ± 0.6</td>
<td>17.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Batch 30</td>
<td>Catalase (237 kDa)</td>
<td>76.9 ± 1.2</td>
<td>16.1 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>
Figure 6.6: *In vitro* release of protein/peptide from PB-A NPs suspended in PB-E thermosensitive gel (20 wt%).
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 was enhanced from 48 kDa to 237 kDa, the difference in DL for PB-C and PB-D was diminished. This may be due to the reduced diffusivity of protein with large 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 comprised of PB-A NPs suspended in thermosensitive gel (20 wt%). Figure 6.6 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 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 the release of lysozyme (~72% in 63 days). It can be explained due to the fact that octreotide has smaller hydrodynamic radius than lysozyme that may have facilitated rapid diffusion of octreotide from composite formulation. The similar trend was observed when we compared the release profiles of lysozyme and IgG-Fab (~24% in 65 days). These results clearly suggest that the hydrodynamic radius of protein molecules plays a crucial role in defining of their release profiles from composite formulations.

However, we have not observed any significant difference between the release profile of octreotide and insulin (~89% in 63 days). It may possible that the difference in molecular weights between octreotide and insulin were not sufficient enough to exert any difference of diffusivity through formulation that led to the similar release profiles. This fact may be true
with smaller molecular weight proteins. There were no noticeable 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 possible that IgG-Fab, IgG and catalase have much less diffusivity through the polymer matrix (due to large molecular weight), thus their release was mainly controlled by polymer degradation. As published in previous reports, degradation of PCL is very slow [204-207, 217], so it was anticipated that PB copolymers composed of PCL blocks may also degrade very slowly. Due to the slower degradation of PB copolymers, release of IgG-Fab, IgG and catalase may be sustained for significantly longer durations.

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 6.6). The Korsmeyer-Peppas model had the best fit based on R² value. The diffusion exponent, n value ranged from 0.513-0.642 for the tested composite formulations. The n-values between 0.43-0.89 indicate anomalous transport (diffusion and degradation controlled) mechanism of protein release.

Enzymatic activity of lysozyme and catalase

Specific enzymatic activity of lysozyme is reported in Table 6.7. Lysozyme activity of freshly prepared solution was found to be 61.4 ± 3.8 (U/mg of protein) ×10³. As described in the Table 6.7, enzymatic activity estimated for released samples were relatively higher than the respective controls. PB copolymers are composed of PEG, a hydrophilic block.
Table 6.6: Coefficient of determination ($R^2$) for various kinetic models for \textit{in vitro} release of octreotide, insulin, lysozyme, IgG-Fab, IgG and catalase.

<table>
<thead>
<tr>
<th>Block copolymers</th>
<th>Korsmeyer-Peppas</th>
<th>Higuchi</th>
<th>Hixson-Crowell</th>
<th>First-Order</th>
<th>Zero-Order</th>
<th>Best fit model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R^2$</td>
<td>$n$</td>
<td>$R^2$</td>
<td>$R^2$</td>
<td>$R^2$</td>
<td>$R^2$</td>
</tr>
<tr>
<td>Octreotide</td>
<td>0.990</td>
<td>0.513</td>
<td>0.983</td>
<td>0.988</td>
<td>0.989</td>
<td>0.949</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.996</td>
<td>0.523</td>
<td>0.988</td>
<td>0.977</td>
<td>0.987</td>
<td>0.901</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>0.994</td>
<td>0.616</td>
<td>0.9926</td>
<td>0.967</td>
<td>0.988</td>
<td>0.937</td>
</tr>
<tr>
<td>IgG-Fab</td>
<td>0.997</td>
<td>0.642</td>
<td>0.996</td>
<td>0.961</td>
<td>0.956</td>
<td>0.966</td>
</tr>
<tr>
<td>IgG</td>
<td>0.998</td>
<td>0.597</td>
<td>0.980</td>
<td>0.996</td>
<td>0.991</td>
<td>0.994</td>
</tr>
<tr>
<td>Catalase</td>
<td>0.992</td>
<td>0.588</td>
<td>0.987</td>
<td>0.932</td>
<td>0.947</td>
<td>0.972</td>
</tr>
</tbody>
</table>
**Table 6.7**: Enzymatic activity of lysozyme estimated in the released samples and controls.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Specific enzyme activity (U/mg) x 10³</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Release samples</td>
<td>Controls</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>61.2 ± 3.4</td>
<td>59.5 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>53.3 ± 4.2</td>
<td>46.8 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>35.7 ± 2.1</td>
<td>26.7 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>63</td>
<td>26.3 ± 3.6</td>
<td>18.9 ± 1.6</td>
<td></td>
</tr>
</tbody>
</table>

**Table 6.8:** Enzymatic activity of catalase estimated in the released samples and controls.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Specific enzyme activity (U/mg) x 10^3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Release samples</td>
<td>Controls</td>
</tr>
<tr>
<td>1</td>
<td>10.8 ± 1.2</td>
<td>10.6 ± 0.5</td>
</tr>
<tr>
<td>15</td>
<td>5.5 ± 0.1</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td>45</td>
<td>3.9 ± 0.2</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>63</td>
<td>3.6 ± 0.2</td>
<td>3.3 ± 0.3</td>
</tr>
</tbody>
</table>
It is anticipated that during NP preparation (W₁/O/W₂ double emulsion), PEG may have been located on the aqueous-organic interface facing towards W₁ and W₂ phase. 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 have reduced the denaturation of protein. However, the biological activity for lysozyme was reduced with the respect to time, as were the controls. This may be due to the fact that protein remained in the release medium for a significant time during the release study. Therefore, storage conditions may have affected the stability of proteins, which will not be the situation at in vivo conditions. During in vivo conditions, proteins released from composite formulation will be immediately absorbed from the vitreous cavity.

According to the results depicted in Table 6.8, enzymatic activity of freshly prepared catalase solution was estimated to be $10.8 \pm 0.2 \times 10^3$. 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 in the formulation may have been protected from hydrolytic degradation. Moreover, the biological activity of the catalase release samples and controls were reduced with the time which may be attributed 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 [178].

**Conclusion**

We have successfully synthesized and characterized novel PB copolymers with different block ratios and block arrangements of PEG/PCL/PLA or PEG/PCL/PGA. These PB copolymers (PLA-PCL-PEG-PCL-PLA and PGA-PCL-PEG-PCL-PGA) were studied for the
preparation of protein/peptide-encapsulated NPs. NP preparation methods were successfully optimized with respect to polymer hydrophobicity, presence of salt (NaCl), drug to polymer ratio, and phase volumes to achieve maximum possible DL. In addition, effect of hydrodynamic radius of protein/peptides on DL and in vitro release behavior were studied. With different arrangement of polymer blocks i.e., PEG-PCL-PLA-PCL-PEG, we have synthesized PB thermosensitive gel which remained in solution phase at room temperature and transformed to gel at body temperature. PB copolymer based composite formulation (PB NPs suspended in PB thermosensitive gel) exhibited constant release for significantly longer duration of time without showing any burst release effect. Moreover, retention of enzymatic activity of lysozyme and catalase in release samples were also confirmed. A biodegradable and biocompatible PB composite formulation comprising protein-encapsulated NPs dispersed in thermosensitive gel can be easily injected intravitreally. This formulation can carry significantly high doses in very limited volume (<100 µL) and can sustain the release of protein/peptides for significantly longer durations without exhibiting burst release. These results clearly suggest that PB copolymer-based protein-encapsulated, formulations can act as a platform therapy for the treatment of posterior segment chronic diseases such as wet-AMD, DR and DME.
CHAPTER 7

IN VIVO OCULAR TOLERABILITY STUDIES OF VARIOUS PENTABLOCK COPOLYMER BASED FORMULATIONS DELIVERED TOPICALLY OR INTRAVITREALLY

Rationale

Vision-threatening disorders such as age-related macular degeneration (wet-AMD), diabetic retinopathy (DR) and diabetic macular edema (DME) are commonly treated with frequent intravitreal injections of anti-VEGF antibodies or fragments thereof. Intravitreal injections are associated with complications such as secondary infection (endophthalmitis), retinal hemorrhage, retinal detachment and more importantly patient non-compliance. Therefore, sustained release therapeutic formulations for ocular posterior segment diseases that can reduce the frequency of intravitreal injection are highly desirable.

This need for sustained-release of anti VEGF therapy has stimulated research on use of thermosensitive in situ hydrogels composed of biodegradable and biocompatible polymers and tissue scaffolds [222-227]. In situ gelling systems offer several advantages over current formulations, including minimal invasiveness (no surgery required) and injectability through small (27 gauge) needles. However, thermosensitive gels alone do not extend drug release sufficiently to reduce the need for frequent injections. Nanoparticulate systems (NPs) have been investigated for the long-term controlled release of anti-VEGF medications. However, NPs alone typically exhibit burst release (initial rapid drug release phase) which can result in dose dependent toxicity. This can be overcome by composite formulations of nanoparticles (NPs) dispersed in a hydrogel, which can provide ocular delivery of therapeutic drug molecules over a longer period of time with a reduced burst effect.
In vitro drug release assays provide limited information regarding in vivo drug release. Moreover, in vitro cell culture based assays only provide toxicity information regarding short-term exposure only. Therefore, in vivo, long-term biocompatibility testing is necessary to establish safety of controlled delivery systems.

In this study, we report the development of novel PB copolymers based controlled release systems and the evaluation of their ocular tolerability. This study provides ocular tolerability of thermosensitive PBC gel was evaluated in rabbits after topical instillation and after a single intravitreal injection of gel, NPs and a composite formulation comprising NPs dispersed in gel. PB copolymer based formulations are under development for the sustained release of biologicals (proteins, antibodies/fragments, RNA) and small molecules (hydrophilic and hydrophobic) for the treatment of ocular diseases.

Materials and methods

Materials

PB copolymers, PB-D and PB-E described in chapter 6, were utilized for the preparation of NPs and thermosensitive gel, respectively. Endotoxin-free water was purchased from Fisher Scientific. Endotoxin estimation kit was procured from GenScript USA Inc. Polyvinyl alcohol (PVA) was obtained from Sigma Aldrich.

Methods

Preparation of sterile, low endotoxin PB thermosensitive gel

Thermosensitive gels were prepared in sterile glassware pre-rinsed with endotoxin free water. In order to prepare low endotoxin PB thermosensitive gel, 20 wt% of PB copolymer was dissolved in endotoxin free (EF) water. Once the polymer was solubilized, 20 wt% aqueous solutions was filtered through 0.2 µm (pore size) filter in aseptic conditions under
laminar flow. The filtrate was analyzed for its gelling behavior and endotoxin levels. Gelling behavior of aqueous solution was confirmed by exposing the solution to 37 °C for 5 min.

**Preparation of low endotoxin PB NPs**

All aqueous solutions utilized during NP preparation were prepared with EF water followed by filter sterilization. NPs were prepared in sterile glassware pre-rinsed with endotoxin free water. PB-D NPs were prepared by $W_1/O/W_2$ double emulsion solvent evaporation method. Briefly, syringe filtered EF water ($W_1$ phase) was added drop-wise in organic phase (PB-D copolymer dissolved in dichloromethane) under constant sonication. The resulting $W_1/O$ primary emulsion was then added drop-wise in the $W_2$ phase (2% PVA) under constant sonication. The $W_1/O/W_2$ double emulsion was diluted with a $W_3$ phase (2% PVA) and vortexed for 5 min. Dichloromethane was then evaporated from multiple emulsions under low pressure. The resulting NPs were centrifuged at 20000 rpm for 30 min at 4 °C. In order to remove residual PVA, NPs were washed two times with EF water followed by centrifugation. Finally, NPs were suspended in 2 mL of EF water and freeze-dried overnight. After freeze-drying, NPs were stored at -20 °C until further analysis. NPs were tested for endotoxin levels. A schematic diagram of NP preparation is illustrated in Figure 7.1.
**Figure 7.1:** Preparation of low endotoxin PB NPs for ocular tolerability studies.
All the aqueous solutions were prepared with EF water followed by filter sterilization. Thermosensitive gel and NPs were prepared in sterile glassware pre-rinsed with endotoxin free water.

**Evaluation of endotoxin levels**

Levels of endotoxins in various formulations were evaluated utilizing an endotoxin assay kit. Limulus Amebocyte Lysate (LAL) endotoxin assay was performed according to the supplier’s protocol under aseptic conditions (laminar flow). Briefly, three test samples were prepared, i.e. thermosensitive gel solution (20 wt%), NP suspension in EF water (5.5 mg/100 µL) and NP suspension in thermosensitive gel solution (20 wt%). A 100 µL aliquot of test or standard solution/suspensions were transferred in vials followed by addition of 100 µL LAL reagent. Mixture was gently stirred for 30 sec, and followed by incubation at 37 °C for 45 min. After incubation, 100 µL of chromogenic reagent was added and gently stirred for 30 sec followed by 6 min incubation at 37 °C. At the end of incubation, 500 µL of color-stabilizer 1, 2 and 3 were added in a defined order. In order to remove NPs from the samples, samples containing NPs were syringe-filtered. Absorbance of samples and standards were estimated at 570 nm utilizing 96-well plate reader.

**In vivo tolerability studies**

Use of animals in this study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved and monitored by the North Carolina State University Institutional Animal Care and Use Committee. Details about animal, housing and environment are described in Table 7.1. Methods along with evaluation parameters for topical drops and intravitreal injections are described below.
**Table 7.1:** Animals, housing and environmental conditions.

<table>
<thead>
<tr>
<th><strong>Species/Strain</strong></th>
<th>Rabbit (Oryctolagus cuniculus)/New Zealand White</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sources</strong></td>
<td>Charles River</td>
</tr>
<tr>
<td><strong>Age Range at First Dosing</strong></td>
<td>Approximately 8 months</td>
</tr>
<tr>
<td><strong>Weight Range at First Dosing</strong></td>
<td>2 - 3 kg</td>
</tr>
<tr>
<td><strong>Identification</strong></td>
<td>Ear tattoo and cage card</td>
</tr>
<tr>
<td><strong>Physical Examination Time</strong></td>
<td>During acclimation</td>
</tr>
<tr>
<td><strong>Caging</strong></td>
<td>Stainless steel; 17 inches wide x 27 inches deep x 15 inches tall or larger, slatted bottoms. No additional bedding.</td>
</tr>
<tr>
<td><strong>Number of animal per cage</strong></td>
<td>1</td>
</tr>
</tbody>
</table>
| **Target Environmental Conditions** | Photoperiod: 12 h light/12 h darkness  
Temperature: 68 ± 2°F |
In vivo ocular tolerability studies were performed in New Zealand White Rabbits. Two batches of filter sterilized low endotoxin thermosensitive gel (FS), one batch of filtered sterilized and gamma irradiated gel (FS+G), one batch of PB-D NPs dispersed in PBS (NP-PBS) (5.5 mg/100 µL) and one batch of PB-D NPs suspended thermosensitive gel (NP-Gel) (5.5 mg/100 µL) were evaluated for topical and intravitreal toxicity.

**Part - I: topical tolerability studies in rabbit eye model**

**Dosing**

Three animals were randomly assigned to each of five study groups (Table 7.2). The left eye (OS) of each animal was treated topically with 35 µL of saline (Balanced Salt Solution, Alcon Laboratories, Fort Worth, TX) and an aliquot (35 µL) of each respective test formulation (i.e. PB thermosensitive gel [FS and FS+G], NPs in PBS [NP-PBS] and NPs in gel [NP-Gel] was administered to the ocular surface of right eye via calibrated pipette. Animals were then allowed to blink several times prior to returning to the cage. Topical tolerability studies were performed by administering 4 doses (each 35 µL) at 15 min intervals followed by an observation period of 24 h. Microscopic ocular irritation was scored in both eyes using the Hackett-McDonald Ocular scoring system at 15, 30, and 60 minutes and 3, 6, 12 and 24 hours after the last topical dose.

**Part - II: tolerability studies after intravitreal injection in rabbit eye model**

**Dosing**

To evaluate ocular biocompatibility of PB copolymer based formulations, 100 µL of each of the five formulations were injected intravitreally in the normal NZW rabbits. The number of animals assigned per group is described in Table 7.3.
### Table 7.2: Animals assigned per group for topical tolerability study.

<table>
<thead>
<tr>
<th>Group #</th>
<th>No. of rabbits per group</th>
<th>Treatment Topical (4 doses; 35 µL each, 15 min apart)</th>
<th>Concentration of polymer</th>
<th>pH of the formulation</th>
<th>Sterility</th>
<th>Measured Endotoxin Levels (EU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>OD: PB Gel (FS); OS:BSS</td>
<td>20 wt%</td>
<td>7.4</td>
<td>Filtered sterilized</td>
<td>BLOQ</td>
</tr>
<tr>
<td>1A</td>
<td>3</td>
<td>OD: PB Gel (FS + G); OS:BSS</td>
<td>20 wt%</td>
<td>7.4</td>
<td>Filtered sterilized followed by gamma irradiation</td>
<td>BLOQ</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>OD: PB Gel (FS); OS:BSS</td>
<td>20 wt%</td>
<td>7.4</td>
<td>Filtered sterilized</td>
<td>BLOQ</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>OD: PB NPs in PBS; OS:BSS</td>
<td>5.5 mg of NPs per 100 µL of PBS</td>
<td>7.4</td>
<td>Non-sterile</td>
<td>0.17 ± 0.04</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>PB NPs in PB Gel (NS); OS:BSS</td>
<td>5.5 mg of NPs per 100 µL of 20 wt% gel</td>
<td>7.4</td>
<td>Non-sterile</td>
<td>0.21 ± 0.01</td>
</tr>
</tbody>
</table>

BLOQ: Below limit of quantification  
OD: Right eye  
OS: Left eye  
FS: Filter Sterilized  
FS+G: Filter Sterilized + gamma Irradiated  
NS: Non-Sterilized  
NPs: Nanoparticles  
PBS: Phosphate Buffered Saline  
PB: PB Copolymer
Table 7.3: Animals assigned per group for tolerability study of intravitreal injection.

<table>
<thead>
<tr>
<th>Group #</th>
<th>No. of rabbits per group</th>
<th>Treatment Intravitreal (100 µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2</td>
<td>Saline</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>PB Gel (FS) (20 wt%)</td>
</tr>
<tr>
<td>1A</td>
<td>6</td>
<td>PB Gel (FS+G) (20 wt%)</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>PB Gel (FS) (20 wt%)</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>PB NPs in PBS (NS) (5.5 mg of NPs per 100 µL of PBS)</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>PB NPs in PB Gel (NS) (5.5 mg of NPs per 100 µL of 20 wt% gel)</td>
</tr>
</tbody>
</table>

FS: Filter Sterilized
FS+G: Filter Sterilized + gamma Irradiated
NS: Non-Sterilized
NPs: Nanoparticles
PBS: Phosphate Buffered Saline
PB: PB Copolymer
Rabbits were tranquilized with ketamine/dexmeditomidine intramuscularly and were prepared by applying 5% betadine solution topically followed by rinsing with sterile saline and application of 0.5% proparacaine HCl. Intravitreal injections (100 µL) of test formulation or saline was done 2 mm posterior to the superior limbus (through the pars plana) using a 25 gauge needle. Special care was taken during the injection to avoid needle contact with the lens. Following the injection, one drop of 0.5% moxifloxacin (Alcon Laboratories, Fort Worth, TX) was instilled topically to the ocular surface.

Parameters to be evaluated

Ocular Examination

Ocular surface morphology, posterior and anterior segment inflammation, retinal changes and cataract formation were evaluated by ocular examination using a slit lamp and indirect ophthalmoscope. Modified Hackett-McDonald ocular scoring (Microscopic Ocular Grading System without fluorescein application) of inflammation was recorded. Parameters such as congestion (0-3), swelling (0-4), discharge (0-3), aqueous flare (0-3), light reflex of iris (0-4), corneal cloudiness affected area (0-4), corneal cloudiness severity (0-4) and pannus and vitreal cells (0-2) were considered to calculate cumulative mean Hackett-McDonald ocular irritation scoring. Ocular examination was carried out for each rabbit to determine pre-dose (pre-study) baseline data, followed by examination during predetermined time intervals. Formulations were evaluated for tolerability studies after topical and intravitreal injections.

Intraocular Pressure

Intraocular pressure (IOP) was measured in both eyes for control and treated groups. IOP was measured at least twice during the acclimation, then immediately following ocular
examination at the time listed above. Briefly, IOP was estimated without use of topical anesthetic. The Tonovet (iCare tonometer, Finland) tip was used to contact the central cornea and record six consecutive measurements. Post-treatment and pre-treatment IOPs were measured during the same time of the day.

Electroretinography (ERG)

Electroretinography was performed prior to injection in all animals, then again at 4 and 16 weeks (in surviving animals). Prior to ERG, all animals were dark adapted for 15 min. A monopolar contact lens electrode (ERG-jet, La Chaux des Fonds, Switzerland) was placed on the cornea to serve as an active electrode and subdermal electrode at the lateral canthus served as the indifferent electrode. A Barraquer eyelid speculum was placed to maintain open eyelids and a subdermal needle electrode was inserted dorsally as the ground electrode. ERGs were elicited by delivering brief flashes at 0.33 Hz with a mini-ganzfeld photostimulator (Roland Instruments, Wiesbaden, Germany) at maximal intensity. For each animal at each testing interval, twenty responses were amplified, filtered, and averaged (Retiport Electrophysiologic Diagnostic Systems, Roland Instruments, Wiesbaden, Germany)

Euthanasia

Rabbits were euthanized at the predefined time intervals. Euthanasia was performed by intravenous injection of an AVMA-approved barbiturate-based agent. Immediately after euthanasia, eyes were enucleated and processed for histopathology. Following eye removal, carcasses were discarded without necropsy.

Ocular Histopathology
Both eyes of each animal were fixed in Davidson’s solution for 24 h, followed by alcohol. Central sections of each globe, including the optic nerve were stained with hematoxylin and eosin stain (H&E stain), and examined by light microscopy.

**Results and discussion**

*Estimation of endotoxin levels in PB thermosensitive gel and PB NPs*

Levels of endotoxins in various formulations were evaluated utilizing Limulus Amebocyte Lysate (LAL) endotoxin assay kit. Both batches of PB thermosensitive gelling solution depicted negligible endotoxin levels (below the limit of quantification) (Table 7.2). PB NPs alone (Group 3), and PB NPs (Group 3) dispersed in PB thermosensitive gel (Group 2) exhibited endotoxin levels of 0.17 ± 0.04 EU/mL and 0.21 ± 0.01 EU/mL, respectively. However, these levels of endotoxins for Groups 3 and 4 are well within the acceptable limit (2 EU/mL) for ocular applications.

*Tolerability study after topical application*

Topical ocular tolerability studies were performed on two batches of thermosensitive gel (20 wt%), PB NPs in PBS buffer, and PB NPs dispersed in thermosensitive gel (20 wt% filter sterilized). These formulations were instilled directly on the ocular surface in right eye of each animal, while left eyes were treated with balanced salt solution (BSS).

*General ocular observation*

No adverse reactions (blepharospasm or evasive action) by the animals were observed during or after the administration of test samples or BSS. Immediately after the application to the left eye, BSS rapidly flowed to the ventral conjunctival cul-de-sacs and disappeared from view.
Figure 7.2: Representative images of rabbit eyes, 6 h post-topical application. (a), (b) and (c) are control eyes for the Groups 1, 3 and 4. (d) PB thermosensitive gel treated rabbit eye (Group 1), (e) PB NPs treated rabbit eye (Group 3) and (f) PB NPs dispersed PB thermosensitive gel treated rabbit eye (Group 4).
Table 7.4: Mean cumulative Hackett-McDonald Ocular Irritation Scores after topical treatment.

<table>
<thead>
<tr>
<th>Group Identification (n/group)</th>
<th>Eye</th>
<th>Pre-Dose 0 h</th>
<th>0.25 h</th>
<th>0.5 h</th>
<th>1 h</th>
<th>3 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (3)</td>
<td>Left (OS)</td>
<td>0</td>
<td>0</td>
<td>0.3 ± 0.6</td>
<td>0</td>
<td>0.3 ± 0.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Right (OD)</td>
<td>0</td>
<td>0.3 ± 0.6</td>
<td>0.7 ± 0.6</td>
<td>0.3 ± 0.6</td>
<td>2.0 ± 0.0*</td>
<td>0.3 ± 0.6</td>
<td>0.7 ± 0.6</td>
<td>0.3 ± 0.6</td>
</tr>
<tr>
<td>1A (3)</td>
<td>Left (OS)</td>
<td>0.3 ± 0.6</td>
<td>0.3 ± 0.6</td>
<td>0.3 ± 0.6</td>
<td>0.3 ± 0.6</td>
<td>0.3 ± 0.6</td>
<td>0.3 ± 0.6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Right (OD)</td>
<td>0.3 ± 0.6</td>
<td>2.0 ± 1.0*</td>
<td>2.0 ± 0.0</td>
<td>1.7 ± 0.6</td>
<td>1.7 ± 0.6</td>
<td>1.3 ± 0.6</td>
<td>1.0 ± 1.0</td>
<td>1.0 ± 1.0</td>
</tr>
<tr>
<td>2 (3)</td>
<td>Left (OS)</td>
<td>0</td>
<td>0.3 ± 0.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Right (OD)</td>
<td>0.3 ± 0.6</td>
<td>2.0 ± 0.0*</td>
<td>1.3 ± 0.6*</td>
<td>1.3 ± 0.6</td>
<td>0.3 ± 0.6</td>
<td>0.3 ± 0.6</td>
<td>0.3 ± 0.6</td>
<td>0.3 ± 0.6</td>
</tr>
<tr>
<td>3 (3)</td>
<td>Left (OS)</td>
<td>0</td>
<td>0.3 ± 0.6</td>
<td>0.3 ± 0.6</td>
<td>0.3 ± 0.6</td>
<td>0</td>
<td>0.3 ± 0.6</td>
<td>0.3 ± 0.6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Right (OD)</td>
<td>0</td>
<td>1.0 ± 1.0</td>
<td>1.3 ± 0.6</td>
<td>0.7 ± 1.2</td>
<td>1.0 ± 1.0</td>
<td>0.3 ± 0.6</td>
<td>0.3 ± 0.6</td>
<td>0</td>
</tr>
<tr>
<td>4 (3)</td>
<td>Left (OS)</td>
<td>0</td>
<td>0.3 ± 0.6</td>
<td>0.3 ± 0.6</td>
<td>0.3 ± 0.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Right (OD)</td>
<td>0</td>
<td>1.3 ± 0.6</td>
<td>1.3 ± 0.6</td>
<td>1.0 ± 1.0</td>
<td>0.7 ± 0.6</td>
<td>1.3 ± 0.6*</td>
<td>1.0 ± 1.0</td>
<td>0.7 ± 0.6</td>
</tr>
</tbody>
</table>

SD = Standard Deviation
Cumulative Observation Score: Average of severity scores for all observations combined (congestion, swelling, discharge, aqueous flare, light reflex (iris), involvement (iris), corneal cloudiness (severity and affected area), pannus and vitreal cells. However severity scores were zero for aqueous flare, corneal cloudiness, pannus and vitreal cells.

* OD: significantly greater than OS (P = 0.033; Wilcoxon test)
PB thermosensitive gel solutions were able to be administered on the corneal surface by pipette where, upon contact with cornea, the solution transformed to clear uniform gel film. Representative images of the control (BSS) and PB formulations treated eyes are depicted in Figure 7.2. PB gel film was observed on the ocular surface through the 1 h examination and a small amount of gel was observed in the conjunctival cul-de-sacs at the 6 h examination. However, application of the PB NPs demonstrated milky opacity on the corneal surface for a few seconds that rapidly dissolved and cleared from the ocular surface.

**Ocular Examination (slit lamp and indirect ophthalmoscope) and Irritation Scores**

Throughout the 24 h post-treatment observations, both BSS treated left eyes and PB formulation treated right eyes exhibited very minimal mean cumulative Hackett-McDonald ocular irritation scores (≤ 2) (Table 7.4). No statistically significant differences in ocular irritation scores were observed between test articles at any time points post-treatment. We have observed statistically significant elevation of ocular irritation scores at several individual time points relative to control treatment (BSS) i.e., Group 1 at 3 h, Group 1A at 0.25 h, Group 2 at 0.25, 0.5 and 1 h, and Group 4 at 6 h. However, cumulative ocular scores were ≤ 2, representing mild and transient congestion (swelling) and conjunctival hyperemia (redness). There was no evidence of intraocular or corneal inflammation. No significant difference in ocular irritation scores between the groups were observed at 24 h post-treatment examination suggesting excellent tolerability of the PB copolymer based formulations for topical ocular applications.

**Tolerability study after intravitreal injection**

**Ocular irritation after single intravitreal injection**
Mildly elevated mean cumulative Hackett-McDonald ocular irritation scores peaked at ~1 h after intravitreal injection and returned to baseline at 7 days (Figure 7.3). There were no significant differences observed in inflammatory scores between the groups except for the group 3 which exhibited elevated inflammation at 6 and 7 days. Animals treated with PB NPs in PBS buffer (Group 3) demonstrated mild signs of uveitis including iris hyperemia and aqueous flare. Animals injected with composite formulations (PB NPs dispersed in PB thermosensitive gel) exhibited no signs of elevated clinical inflammatory scores relative to saline treated eyes or PB gel treated eyes. No signs of inflammation have been subsequently observed in eyes examined up to 4 months after injection.

**Other ocular examination findings**

Focal posterior cortical cataract was observed in one of the rabbit (group 1) at day 1 after the injection was observed. However, this lesion was not changed in size over the course of study. It was anticipated that formation of cataract was most likely from the injection procedure and not associated with the PB gel itself. Hence, it was not considered as a sign of toxicity. We have not observed any signs of vitreous inflammation or toxicity, damage of retinal or optic nerve, or lens toxicity (opacity or cataract) (Figures 7.4, 7.5 and 7.6).

**Intraocular pressure (IOP)**

All rabbits exhibited IOP in acceptable range, and no consistent elevation or decrease of IOP was noted. One exception was in group 3, where rabbits demonstrated significantly (P = 0.03) reduced IOP at day 7 after the injection relative to the saline or PB-gel treated animals. This result was consistent with observation of with the clinical inflammation noted slit-lamp examination (Figure 7.7).
Figure 7.3: Mean +/-SD Cumulative Hackett-McDonald Irritation Scores.
Figure 7.4: Images of rabbit eyes taken after single intravitreal injection (100 µL) of PB thermosensitive gel, (a) day 1, (b) day 21, (c) day 42, (d) day 49, and (e) day 98.
Figure 7.5: Images of rabbit eyes taken after single intravitreal injection (100 µL) of PB NPs dispersed in PBS, (a) day 1, (b) day 21, (c) day 35, (d) day 42, and (e) day 77.
Figure 7.6: Images of rabbit eyes taken after single intravitreal injection (100 µL) of PB NPs dispersed in PB thermosensitive gel, (a) day 1, (b) day 21, (c) day 35, (d) day 42, and (e) day 77.
Figure 7.7: Intraocular pressure (IOP) estimated after single intravitreal injection.
Figure 7.8: Electroretinography performed after single intravitreal injection.
Table 7.5: Ocular histopathology of rabbit eyes after single intravitreal injection of various PB copolymer based formulations.

<table>
<thead>
<tr>
<th>Group</th>
<th>Weeks after injection</th>
<th>Anterior segment inflammation</th>
<th>Posterior segment inflammation</th>
<th>Lens damage</th>
<th>Retinal damage</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group 1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Few mononuclear cells in vitreous near lens</td>
</tr>
<tr>
<td>Group 1A</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Few neutrophils in anterior vitreous</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>Neutrophils in anterior vitreous, posterior lens rupture</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group 2</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Focal area of lens capsule rupture and lens fiber change</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Few neutrophils in anterior chamber and ICA</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Focal area of lens capsule break and lens fiber change</td>
</tr>
<tr>
<td>Group 3</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Epithelial downgrowth, lens rupture, Pyogranulomatous infiltrate</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group 4</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Epithelial downgrowth at injection site</td>
</tr>
</tbody>
</table>

- = none  
+ = mild  
++ = moderate  
+++ = severe
**Electroretinography**

The pre-study and weeks 1, 4, add 16 post injection ERGs were normal in each rabbit. No significant differences in a-wave and b-wave implicit times or amplitudes before and after treatment (Figure 7.8) were observed, suggesting a lack of retinal toxicity with the injected polymers up to 4 months.

**Ocular histopathology**

Ocular histopathology was performed at weeks 1, 4 and 16 after intravitreal injection. Results reported in Table 7.5 indicated that in most eyes no evidence of inflammation or toxicity associated with the injection procedure or test articles were observed. Two eyes exhibited mild anterior segment/anterior vitreous inflammation. However, we have observed injection procedure associated damage and inflammation in a few eyes, including lens trauma and wound epithelial downgrowth. These changes appear to be associated with the injection procedure in sensitive rabbit eyes rather than the test articles. Only one animal of group 3 (PB NPs in PBS) exhibited severe posterior segment inflammation at week 1 after injection. These results are consistent with the results of ocular irritation score and reduced IOP observed in the same group of animals. Based on these findings, it appears that the test articles are well tolerated by the rabbit eyes, without evidence of overt inflammation or histopathologic evidence of tissue damage indicating excellent biocompatibility of PB copolymer based formulations. However, changes associated with injection procedure are relatively common and may cause most of the inflammatory and tissue damage observed (both histologic and clinical).
Conclusion

We have prepared endotoxin-free thermogelling polymer solutions and have tested these with topical application to rabbit eyes. In addition we have tested thermogelling solution with and without PB NPs. Topical application of 4 consecutive doses (35 µL each) at intervals of 15 min produced no signs of irritation. No adverse reactions, such as evasive action or blepharospasm were observed during or after the application suggesting adequate tolerability for topical ocular applications. Minimal mean cumulative Hackett-McDonald ocular irritation scores of ≤2 were observed for all the formulations indicating mild and transient conjunctival hyperemia (redness) and congestion (swelling). Low ocular irritation scores up to 4 weeks demonstrate that PB copolymer NPs dispersed in thermogelling solution are well tolerated. No significant difference in IOP was observed between saline treated or PB copolymer formulation treated eyes. ERG results showed no changes in retinal functions in either control or test groups. Moreover, no sign of inflammation or cataract formation during the treatment period was observed. These results indicate that PB copolymer based formulations are biodegradable and biocompatible, and well suited for ocular application, both topically or by intravitreal injection. These polymers can be successfully used for the development of controlled release systems for ocular delivery of therapeutic proteins.
CHAPTER 8
SUMMARY AND RECOMMENDATIONS

Summary

Development of sustained release formulation for the protein therapeutics in the treatment of posterior segment diseases such as wet-AMD, DR and DME, is a very challenging task for the formulation scientists. The ideal formulation for the intravitreal delivery should possess following characteristics, (a) high drug loading in small volume (≤ 100 µL) that lasts up to 6 months or more, (b) provide constant release (zero-order release) throughout the release period without any burst effect, (c) easy to administered such as injectable system and not implants, (d) ensure stability of protein/peptide, (e) biodegradable and biocompatible, and (f) the time required for biodegradation of formulation should not be more than 1.5 times the release period.

In order to achieve this goal, we have synthesized novel biodegradable and biocompatible PB copolymers. PB copolymers are composed of various FDA approved polymeric blocks such as PEG, PCL, and PLA/PGA. Each block plays an important role such as presence of PEG helps to improve stability of NPs by reducing NP aggregation, and also helps to escape phagocytosis by macrophages resulting in improved half-life. PCL is a slow degrading semi-crystalline polymer which improves protein loading in NPs and also sustains drug release for longer duration of time. Such a slow degradation is also not advantageous particularly for intravitreal injections. It is important for formulation scientists to synchronize polymer degradation profiles with the drug release profiles in order to avoid accumulation of empty formulation in the limited vitreous cavity. Previous reports suggest that poor degradation of PCL is attributed to its crystalline nature, hence reduction in the crystallinity of PCL may improve its hydrolytic and enzymatic degradation. Covalent conjugation of
PLA/PGA to PCL chains can significantly reduce crystallinity resulting in faster degradation of PCL [203, 219]. PB copolymers were utilized to prepare thermosensitive gel or NPs.

Firstly, we have synthesized and characterized various TB (PCL-PEG-PCL, B-A-B) and PB (PLA-PCL-PEG-PCL-PLA (C-B-A-B-C) and PEG-PCL-PLA-PCL-PEG (A-B-C-B-A)) copolymer based thermosensitive gelling polymers. Arrangement of polymer blocks, molecular weight and presence of PLA exhibited significant effect on sol-gel transition curve and kinematic viscosity of respective aqueous solutions. IgG-loaded PB thermosensitive gels exhibited sustained release up to ~20 days. Results of release kinetics suggested Korsmeyer-Peppas as a best fit model and diffusion exponent values (n value) were ranging from 0.272-0.386 for all gelling polymers indicating diffusion controlled release of IgG. Moreover, secondary stability of released IgG was confirmed by CD spectroscopy. PB copolymers with A-B-C-B-A block arrangements demonstrated noticeably lower kinematic viscosity of aqueous solution at 25 °C than the TB (B-A-B) copolymers and other PB copolymers with C-B-A-B-C block arrangements suggesting easy to handle during injection. Presence of PLA block has significantly reduced crystallinity of PB copolymers (C-B-A-B-C and A-B-C-B-A). Hence, it was anticipated that PB copolymers will have faster rate of degradation relative to PCL-PEG-PCL based TB copolymers. The difference in results of viscosity and sol-gel transition has been explained by two different processes of micellization for A-B-C-B-A and B-A-B or C-B-A-B-C types of copolymers. Cell viability and biocompatibility studies suggested that PCL, PLA and PEG based block copolymers are compatible with various ocular and macrophage cell lines. In our first approach, we were able to develop sustained release, biocompatible and biodegradable polymeric formulation which can be administered with semi-invasive technique. However, thermogelling system exhibited sustained release up to only ~20
days, which was shorter than our target profile (4-6 months). Moreover, it also demonstrated significant burst release.

Therefore in our second approach, we have utilized PB copolymers for the preparation of protein-encapsulated NPs. We have synthesized and evaluated series of PB copolymers with significantly high molecular weights and utilized them for the preparation of NPs. PB copolymers with different block arrangement and molecular weights were successfully synthesized by ring-opening bulk polymerization and characterized by $^1$H-NMR, gel permeation chromatography (GPC) and X-ray diffraction (XRD) spectroscopy. We have prepared IgG-loaded (a model protein) PB NPs and studied the effects of molecular weight, polymer composition (PEG/PCL/PLA or PEG/PCL/PGA) and isomerism (PLA with L or DL isomers) on various formulation parameters such as EE, DL and in vitro release profile. Results demonstrated that crystallinity of PB copolymers can be easily modulated by altering the ratio of PLA/PCL or PGA/PCL. Moreover, molecular weight, crystallinity and copolymer composition exhibited significant effect on EE, DL and in vitro release kinetics. PB copolymers composed of PLA with D,L-lactide demonstrated EE (~63%), and DL (~5.8%) with sustained release up to ~60 days which were significantly higher than PB copolymers comprising PLA (L-lactide) or PGA. Secondary structure of released IgG was confirmed by CD spectroscopy. In vitro cytotoxicity, cell viability and inflammatory studies performed on human retinal pigment epithelial cells (ARPE-19) and/or macrophage cell line (RAW-264.7) established biocompatibility of PB copolymers for ocular applications. However, PB NPs exhibited significant burst release within first two days which may be attributed to surface adsorbed protein. Moreover, poor DL was also an issue which was needed to be resolved. In
addition, upon intravitreal injection, NPs may distribute throughout the vitreous (floater effect) and obstruct patient’s vision which may eventually leads to patient’s non-compliance.

In order to resolve the problem of burst release and floater effect, we designed a composite formulation. The composite formulation was comprised of protein-encapsulated PB NPs suspended in PB thermosensitive gel. A novel approach had successfully diminished burst release effect and exhibited nearly zero-order release throughout the release period. This behavior may be due to the fact that when NPs were suspended in gel matrix, entangled polymer chains of thermosensitive gel may serve as an additional diffusion barrier for the surface adsorbed drug. The polymer matrix might deter the dumping of surface adsorbed dose eliminating burst effect and avoiding any possibility of dose dependent toxicity which may occur due to the initial burst release. Simultaneously, we have also hypothesized that proteins with similar molecular weights behave very identical in same polymer matrix. Hence, we have prepared and characterized PB NPs loaded with BSA (66 kDa), IgG (150 kDa) and bevacizumab (150 kDa). Our results demonstrated not significant difference in EE, DL and in vitro release profile of IgG or bevacizumab from PB NPs. However, these results were significantly different than BSA-encapsulated PB NPs. Moreover, cell viability, cytotoxicity and biocompatibility studies performed with PB copolymers revealed non-toxic and biocompatible nature of PB copolymers. Results of CD spectroscopy, cell migration and cell proliferation assays confirmed structural and biological activity of released proteins. In situ thermosensitive gelling solution immediately transforms to gel at body temperature entrapping protein-loaded PB NPs that inhibits PB NP dispersion in the vitreous fluid and localizes them in the gel matrix. Hence, by developing a composite formulation, we were able to eliminate burst release, achieved nearly zero-order drug release and also eliminated possibility of the
floater effect. However, in order to deliver large dose in very limited injection volume, the most important challenge was to improve DL. We were also not able to sustain the release for more than ~60 days. From our previous results we learnt that hydrophobicity of polymer have significant effect on sustaining the release of proteins.

In our last approach we have synthesized most hydrophobic PB copolymers to sustain the release for significantly longer duration and we have optimized NP preparation parameters to achieve maximum possible DL. Most hydrophobic PB copolymers were synthesized with two different block arrangements i.e., PLA/PCL/PEG and PGA/PCL/PEG with very high molecular weight. NP preparation method was successfully optimized with respect to polymer hydrophobicity, presence of salt (NaCl), drug to polymer ratio, and phase volumes to achieve high DL. With optimized NP preparation methods, we were able to achieve more than 15% of loading for IgG-Fab, IgG and catalase, and ~8% of loading for octreotide, insulin and lysozyme in PB NPs. PB NPs encapsulating different proteins were also evaluated for their sustain release behavior. All the NPs depicted negligible or no burst release phase. We have observed significantly rapid release of octreotide (~93% in 63 days) from composite formulation relative to the release of lysozyme (~72% in 63 days). It can be explained due to the fact that octreotide has smaller hydrodynamic diameter than lysozyme which may have facilitated rapid diffusion of octreotide from composite formulation. The similar trend was observed when we compared the release profiles of lysozyme and IgG-Fab (~24% in 65 days). These results clearly suggest that the hydrodynamic radius of protein molecules plays a crucial role in defining of their 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 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 be true with 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 NPs dispersed in thermosensitive gel. It may possible that IgG-Fab, IgG and catalase have very less diffusivity through polymer matrix (due to large molecular weight) and hence their release was mainly controlled by degradation of polymers. With the projected release profiles for IgG-Fab, IgG and catalase, we can easily achieve sustain release for more than 6 months. We have also confirmed enzymatic activity of lysozyme and catalase in the release samples with their respective enzymatic activity assays.

Finally, we have evaluated in vivo tolerability of in situ thermosensitive gel alone, PB NPs alone and a composite formulation in rabbit eye model. Intravitreal injection of in situ thermosensitive gel and composite formulation demonstrated immediate transformation from solution to hydrogel. All the formulations were easily injectable through a 27 gauge needle without showing any back pressure. Moreover, as anticipated, PB NPs were dispersed throughout the vitreous immediately after intravitreal injection and remained dispersed even after 56 days. However, intravitreal injection of composite formulation formed a depot entrapping PB NPs inhibiting NP dispersion in vitreous fluid. In situ thermosensitive gel (alone) disintegrated and dissolved in vitreous fluid within 56 days after intravitreal delivery indicating the biodegradable nature of PB copolymers. Rabbit eyes exhibited no signs of toxicity (inflammation, cataract or change in ocular pressure) against any of the PB copolymer-based formulations. Similar formulations were also investigated for their topical applicability.
Even after 4 doses of topical instillation, no signs of irritation or inflammation were observed in rabbit eyes.

In this project, we were successful to develop novel PB based composite formulation for the intravitreal delivery of protein therapeutics. This formulation exhibited all the desired properties of ideal formulation such as, biodegradability, biocompatibility, ability to carry large dose in limited volume, depicted more than 6 month of release, exhibited nearly zero-order release without burst effect, easy injectability, and also ensured stability of proteins/peptides.

**Recommendations**

According to our results, proteins with similar molecular weights behave very similarly during NP preparation with same PB copolymers. Hence, they exhibit very similar EE, DL and *in vitro* release profile. Our optimized sustained delivery formulation can be utilized with fine tuning to develop a formulation with same molecular weight proteins. PB copolymers utilized for NP preparation are composed of PLA/PCL/PEG and PGA/PCL/PEG with block arrangements of PLA-PCL-PEG-PCL-PLA and PGA-PCL-PEG-PCL-PGA. These polymers demonstrated very slow release rate of IgG-Fab (2-2.5 µg/day). Therefore, these polymers are suitable for very potent drugs or to conditions where a lower rate of release is required for a longer duration of time (about 6-10 months). However, these polymeric systems are not useful where higher release rate with high DL is required. Therefore, there is a need to develop a formulations that can provide higher release rate for 4-6 months. According to results, release of IgG-Fab, IgG and catalase (high molecular weight proteins) from the composite formulation is mainly govern by degradation of polymers. Hence, by improving the hydrolytic degradation of PB copolymers, a higher rate of protein release can be achieved. This goal can be achieved
by changing the order of block copolymers to PCL-PLA-PEG-PLA-PCL or PCL-PGA-PEG-PGA-PCL. PEG is a non-degradable polymer whereas PCL is very slow degrading polymer. PLA and PGA are rapidly degrading polymer blocks. In case of PLA-PCL-PEG-PCL-PLA and PGA-PCL-PEG-PCL-PGA terminals blocks degrades very rapidly leaving slow degrading TB copolymer (PCL-PEG-PCL). However, for the new sequence of PB copolymers (PCL-PLA-PEG-PLA-PCL or PCL-PGA-PEG-PGA-PCL) center blocks (PLA/PGA) degrades rapidly, leaving small fragments of block copolymers comprised of homo polymers (PCL or PEG), diblock copolymers (PCL-PLA or PLA-PEG) or TB copolymers (PLA-PEG-PLA or PCL-PLA-PEG). Resulting diblock or TB copolymers may eventually degrade to homopolymers of PEG or PCL. Moreover, this arrangement of blocks will significantly reduce the crystallinity of PB copolymers which will further enhance the rate of polymer degradation. Rapid disintegration of PB copolymers can improve the rate of protein release from the composite formulation. According to the results observed during in vivo studies, the thermosensitive gelling polymer degraded very rapidly relative to PB NPs. Faster degradation of gelling polymer may allow PB NPs to escape from the depot to some extent. Free NPs may interfere with a patient’s vision, leading to non-compliance. Uptake of these NPs in retinal epithelial cells could be enhanced by functionalizing the NP surface with targeting agents such as folate or biotin. Surface modified PB NPs might be recognized by folate receptor or sodium-dependent multivitamin transporter (SMVT) located on the apical site of retinal pigment epithelial cells. Hence via surface modification approach, free NPs may be diverted from the vitreous fluid to the target site. The composite formulation approach may serve as a platform technology for the delivery of siRNA, peptides, proteins and antibodies in the treatment of
ocular diseases. This technology may not be limited to ocular applications but may be used for any chronic diseases where sustained delivery of macromolecules is required.
APPENDIX

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VITA

Sulabh P. Patel was born on 3rd June, 1983, in Ahmedabad, Gujarat, India. He completed his Bachelor of Pharmacy from Hemchandracharya North Gujarat University in 2004 and received his Master of Science degree with the specialization in Pharmaceutical Analysis (2006) from National Institute of Pharmaceutical Education and Research (NIPER), S.A.S. Nagar, Mohali, Punjab, India. After completion of his M.S. degree, he joined the Bioanalytical division of Cadila Pharmaceuticals, India and worked as Research Associate until August, 2007.

To pursue a Ph.D. degree, Sulabh Patel initiated Doctoral study in the Interdisciplinary Ph.D. program at UMKC in January, 2009. He is an active member of American Association of Pharmaceutical Scientists (AAPS), Association of Research in Vision and Ophthalmology (ARVO) and Pharmaceutical Sciences Graduate Student Association (PSGSA). He served as the Secretary of American Association of Pharmaceutical Scientists (AAPS) student chapter, UMKC, for two consecutive academic years (2010-11 and 2011-12). He received the first prize at Interdisciplinary community of scholar poster presentation, April 2011. He completed his doctoral studies in January, 2014 under the guidance of Dr. Ashim K. Mitra. He has authored/co-authored several peer reviewed publications and book chapters, and has also presented his work in several annual national conferences.