Controlled Delivery of Salmon Calcitonin Using Thermosensitive Triblock Copolymer Depot for Treatment of Osteoporosis

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ABSTRACT: Osteoporosis is a common metabolic bone disorder associated with fragility and bone fracture. Worldwide, osteoporosis results in more than 8.9 million fractures annually. Additionally, steroid treatments can cause osteoporosis as a side effect. Salmon calcitonin (sCT) is injected daily for those on steroid treatments as a means to prevent and treat osteoporosis side effects. Frequent dosing is inconvenient, uncomfortable, and often leads to compliance issues. Our objective was to develop a monomethoxy poly(ethylene glycol) (mPEG) and poly-lactic-co-glycolic acid (PLGA) thermosensitive triblock copolymer (mPEG−PLGA−mPEG)-based controlled release delivery system at an increased lactide to glycolide ratio (3.5:1, 4.5:1, and 5:1) to deliver sCT in its active conformation in a controlled fashion for a prolonged period following a single subcutaneous injection. Increasing lactide to glycolide ratio increases hydrophobicity of the PLGA block, which slows degradation of copolymer, thereby prolonging release and reducing burst release. Proton nuclear magnetic resonance spectroscopy and gel permeation chromatography confirmed structural composition and polydispersity index, respectively. Critical micelle concentration of the copolymer was 25 μg/mL. The delivery system was biocompatible as determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability assay. Moreover, the copolymeric system maintained sCT in a conformationally stable form for the entire duration of storage and release.

1. INTRODUCTION

Osteoporosis, derived from the Greek terms for “porous bones”, is one of the most common metabolic bone disorders characterized by low bone mass.1 In this disease, silent and progressive loss of bone tissue greatly reduces the density and quality of bones. Consequently, the bones become more porous and fragile with an increased susceptibility to painful fractures resulting in substantial morbidity. The most common fractures associated with osteoporosis occur at the hip, spine, and wrist.2−3 Risk factors chiefly include genetics, old age (>50 years), menopause, low body weight, family history of osteoporosis, history of fracture as an adult, history of hormone and autoimmune disorders, inactive lifestyle, lack of calcium and vitamin D, cigarette smoking, and excessive alcohol consumption.4 Certain medications such as steroids, anticonvulsants, anticoagulants, antimitabolites, proton-pump inhibitors, thiazolidinediones, and L-thyroxine have also been associated with increased risk of osteoporosis by different mechanisms.5−11 Worldwide, one in three women and one in five men are at risk of an osteoporotic fracture. In US adult population of age 50 years and older, osteoporosis and low bone mass affect approximately 53.6 million people (54% of the population).12 In addition to considerable pain and disability, osteoporotic bone fractures take a huge personal and economic toll on a person and their family. Elderly patients can develop pneumonia and pulmonary embolism due to prolonged bed rest following a painful fracture.13

The goal of treatment for osteoporosis is prevention of bone fractures by reducing bone loss or preferably by increasing bone density and strength. Antiresorptive drugs such as bisphosphonates are most commonly used in clinical practice. Bisphosphonates inhibit osteoclastic bone removal thus increasing bone density, however, use of these drugs is associated with severe acute and long-term side effects, which limit their long-term use and patient compliance.14 Hormone replacement therapy (HRT) in post-menopausal women has been shown to prevent bone loss, increase bone density, and prevent bone fractures mainly due to the chondro-protective effect of estrogen. However, because of the increased risks of heart attack, stroke, venous blood clots, and breast cancer.
Calcitonin is an antiresorptive hormone naturally produced by the parafollicular cells of thyroid gland. It is involved in calcium and phosphorus metabolism and shows a calcium-lowering effect by counteracting PTH. PTH acts to increase the concentration of calcium in blood, owing to increased bone resorption by altering gene expression in osteoblasts. In bones, calcitonin almost exclusively targets calcitonin receptors on osteoclasts interfering with their differentiation from precursor cells, reducing motility and inducing retraction by multiple inhibitory mechanisms. Calcitonin is frequently used in the treatment of several bone-related disorders such as hypercalcemia, Paget’s disease, and osteoporosis. In osteoporosis, calcitonin reduces bone resorption and significantly reduces bone pain, a very common symptom of osteoporosis. Clinically, synthetic or recombinant salmon calcitonin (sCT) is widely used because it has 50% sequence homology to human calcitonin, meanwhile significantly reduces bone pain, a very common symptom of osteoporosis. Clinically, synthetic or recombinant salmon calcitonin (sCT) is widely used because it has 50% sequence homology to human calcitonin, meanwhile demonstrating 40–50 times higher potency than human calcitonin because of its higher affinity toward human calcitonin receptor.

In practice, calcitonin or sCT is administered by subcutaneous, intramuscular, or intranasal routes, daily or multiple times per week, depending on the severity of bone loss. However, frequent administration produces discomfort and reduces patient compliance which negatively affects treatment adherence, thus resulting in treatment gaps. Calcitonin can be administered through the nasal route, however, its bioavailability is only ~25% compared to intramuscular calcitonin. Additionally, intranasal calcitonin is associated with the risk of nosebleeds, runny nose, and other nasal irritations. Oral formulations have been tested, but results demonstrate compromised bioactivity, owing to degradation by enzymes within the digestive tract. Consequently, controlled release delivery systems for sustained delivery of biologics prompted development of subcutaneous implants. In situ gel forming implants consisting of biodegradable polymers became popular for their ease of administration, lack of surgery for implantation and removal, and biocompatibility.

Here, we present the formulation development of a thermosensitive triblock copolymer-based delivery system releasing sCT in its active conformation in a controlled fashion for a prolonged period following a single subcutaneous injection. In the current study, triblock thermosensitive copolymer monomethoxy poly (ethylene glycol)–poly lactic-co-glycolic acid (mPEG–PLGA–mPEG) has been investigated for controlled release of sCT for an extended period of time. Triblock copolymers with increasing ratio of lactide (LA) to glycolide (GA) (3:5:1, 4:5:1, and 5:1) were synthesized to optimize hydrophobic and hydrophilic characteristics of the copolymer. Previous work in our lab has explored LA to GA ratios up to 3:1, and the promising results observed motivated us to explore the possible benefits of increasing LA content beyond the 3:1 ratio. Phase transition temperature of copolymers was determined using the tube-inversion method. The copolymer with physiologically relevant transition was characterized further using proton nuclear magnetic resonance spectroscopy (1H NMR) to determine structural composition and gel permeation chromatography (GPC) to determine average molecular weight and polydispersity index (PDI). Critical micelle concentration (CMC) was determined using pyrene as the fluorescent hydrophobic probe. Two concentrations of copolymer formulation were further investigated for in vitro biocompatibility, release, and stability at physiological and storage conditions.

2. RESULTS
2.1. Synthesis and Characterization of Thermosensitive Triblock Copolymers. Thermosensitive triblock copolymer mPEG–PLGA–mPEG with LA to GA ratio 3.5:1, 4.5:1, and 5:1 were synthesized using ring opening polymerization followed by diblock condensation. The sol to gel transition temperature of the copolymers was tested using the tube inversion method. This method allows the determination of temperature at which the copolymer undergoes complete transition from solution to gel form. Copolymers that transition at or below body temperature (37 °C) but remain a solution at room temperature (~27 °C) are physiologically relevant for in situ depot formation. In this study, the transition temperatures were observed to be greater than 40 °C for copolymers with LA/GA, 3.5:1 and 4.5:1. However, copolymer mPEG–PL5GA1–mPEG (5:1, LA/GA) transitioned to gel form at 36 °C and was selected for further characterization. Thereafter, to confirm quick transition from solution to gel, a fresh sample of copolymer was exposed for 30 s to physiological temperature. As illustrated in Figure 1, the mPEG–PL5GA1–mPEG copolymer (LA/GA, 5:1) copolymer at 30 and 40% w/v aqueous concentrations (30 and 40% w/v) transitioned successfully from sol to gel in 30 s at 37 °C. Further polymer characterization via 1H NMR and GPC was only investigated for the LA/GA, 5:1 copolymer, given that it was the only candidate that transitioned within a physiologically relevant temperature. 1H NMR spectra of the mPEG–PL5GA1–mPEG copolymer confirmed the structure of the synthesized mPEG–PLGA–mPEG triblock copolymer. The polymer demonstrated desired LA to GA ratios corresponding to integrals of peaks.
spectra detected peaks at 1.55, 3.38, 3.65, 4.3, 4.8, and 5.2 ppm corresponding to the CH$_3$ of LA, CH$_3$ of mPEG end group, CH$_2$ of mPEG, CH$_2$ between PLGA and mPEG, CH$_2$ of GA, and CH of LA, respectively (Figure 2).

Molecular weight distribution of the mPEG−PL$_5$GA$_1$−mPEG (LA/GA, 5:1) copolymer was determined using GPC size determination and to provide evidence of the homogeneity of the copolymer (PDI). Number average molecular weight ($M_n$) and weight average molecular weight ($M_w$) of the copolymer were found to be 2950 and 4180 Da, respectively, while PDI was found to be $\sim 1.42$ (Figure 3).

CMC of the mPEG−PL$_5$GA$_1$−mPEG (LA/GA, 5:1) copolymer was determined using the pyrene fluorescence probe method and was found to be 25 $\mu$g/mL (Figure 4). Transition from solution to gel is based on the hydrophobic effect and ability of the polymer to arrange itself into an ordered micellar structure. Low CMC value supports the amphiphilic structure of the copolymer that organizes into fairly stable micelles at relatively low concentration.

2.2. In Vitro Biocompatibility Assay. In vitro biocompatibility of the mPEG−PL$_5$GA$_1$−mPEG (LA/GA, 5:1) copolymer was evaluated by testing different concentrations of the copolymer in HEK 293 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. Biocompatibility in HEK 293 is widely considered necessary to support cyto-compatibility in vivo and assess adverse reactions of the sample. Compared to control, the viability of cells incubated with copolymer samples was found to be higher than 80% when tested up to a concentration of 1 mg/mL (Figure 5). However, increasing the copolymer
concentration beyond 1 mg/mL reduced cell viability. The IC$_{50}$ of the copolymer was found to be >10 mg/mL, which suggests high cyto-compatibility of the copolymer.

2.3. In Vitro Release Profile of Salmon Calcitonin.

Formulations composed of sCT incorporated in the mPEG–PL$_5$GA$_{1}$–mPEG (LA/GA, 5:1) thermosensitive triblock copolymer (30 and 40% w/v) were easily injectable using a 25 G syringe and transitioned instantaneously into a gel upon incubation at 37 °C. Percent cumulative release of sCT from thermosensitive copolymer formulations were studied in vitro. In order to be most effective, the release of therapeutics from a controlled release delivery system should follow zero-order kinetics so that a constant level of the drug is maintained continuously in circulation and hence produce sustained action. In vitro release profile mimics what can be expected to be seen in vivo, which allows for optimization needed prior to in vivo studies. Factors such as burst release, release rate, and complete release are important in optimization of a sustained release formulation based on the drug’s therapeutic index, toxicity profile, and mode of action. Looking at two concentrations of copolymer formulations allows us to optimize the formulation to achieve the best release profile. Higher concentrations of copolymer within the formulation will result in slower release over time because of a few factors such as slower degradation rate and increased viscosity through which therapeutics would need to overcome for the release from diffusion to occur. An initial burst release of sCT at 10.6 ± 0.58 and 7.7 ± 3.8% from the 30 and 40% formulations, respectively, was observed. Burst release was followed by a steady release for up to 49 and 70 days, respectively, for the two formulations and amounted to a cumulative sCT release of 103.2 ± 4.64 and 106.4 ± 6.16% for the 30 and 40% formulations (Figure 6). A low burst, such as that shown in this

![Figure 6. Percent cumulative release of sCT in vitro from mPEG–PL$_5$GA$_{1}$–mPEG (LA/GA, 5:1) thermosensitive triblock copolymer (30 and 40% w/v). Data represent the mean ± SD (n = 4).](image)

which can diffuse out, giving the initial burst release. This is followed by a slow controlled release, owing to controlled breakdown of the polymer and diffusion of therapeutic through the copolymer matrix, which appears as the second phase of release. In this study, optimization of LA to GA ratio has minimized biphasic characteristic of the sCT release profile, and this release best fits zero-order release, which is desirable for depot-based delivery systems. This also reiterates the importance of formulation optimization such as changes in the copolymer composition, which would further help in optimizing the overall release profile of therapeutics through such copolymeric depot-based delivery systems.

2.4. Stability of Salmon Calcitonin Released at Physiological Temperature.

Released sCT must be in its native conformation in order to interact with its receptor and demonstrate bioactivity. Circular dichroism (CD) spectra of sCT released from copolymer depot at 37 °C were examined at 30 and 30 days, showed characteristic minima at ~205 nm, corresponding to the freshly prepared sCT (Figure 7A). Secondary structure analysis of freshly prepared sCT and sCT released from copolymeric formulation is reported in Table 1A. This analysis of sCT released from the copolymer supports its protection from denaturation inside the polymer depot and retention of its bioactive conformation upon release. Previous work at our laboratory using CD, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy, and high-performance liquid chromatography (HPLC), to evaluate the stability and bioactivity of sCT, has shown that PEG/poly lactic acid/PLGA-based copolymer systems are able to protect incorporated protein/peptide from denaturation and release them in a biologically active form.\(^{35}\)

2.4.2. Stability of Salmon Calcitonin inside the Gel during Storage at 4 °C.

Preparation and storage of therapeutics play a large role in the assessment of its suitability for future development and use in the clinics. Stability of sCT incorporated in the gel depot was assessed to make sure that the structure of the protein remains unaltered during storage at 4 °C. CD spectra of sCT extracted from the copolymer depot stored at 4 °C, at 15 and 30 days, showed minima at 208 and 222 nm, which is typical of an $a$-helix structure and characteristic of native conformational structure of sCT in the presence of an organic solvent, as compared to the standard solution comprising freshly prepared sCT in phosphate buffered saline (PBS)/acetonitrile (ACN), 1:1 v/v (Figure 7B). Secondary structure analysis of standard and sCT extracted from stored copolymer depots is reported in Table 1B. These results support the expectation that this formulation can be prepared and stored for at least one month before use. For most purposes, the protein will be incorporated into the polymer and stored under refrigeration until used. Manufacture of formulation that is ready for injection would add to the ease of use in comparison to current conventional methods.

3. DISCUSSION

Controlled release of proteins and peptides in a structurally stable form has been the focus of several investigations over the past decades. Treatment of osteoporosis using controlled release of antiresorptive peptide sCT has been proposed in this study using thermosensitive, triblock copolymer-based delivery system. Thermosensitive triblock copolymer mPEG–PLGA–mPEG was synthesized using ring opening polymerization followed by diblock condensation. The novelty of this study lies in how the LA to GA ratios were varied in the PLGA block.
to optimize hydrophobic and hydrophilic characteristics of the copolymer expanding on our previous work with ratios up to 3:1 LA to GA.\textsuperscript{35} mPEG is hydrophilic and the molecular weight can be varied to increase or decrease the hydrophilic nature of the copolymer.\textsuperscript{36} The PLGA block is hydrophobic with LA being more hydrophobic than GA.\textsuperscript{37} Therefore, increasing the block size and/or LA to GA ratio can influence the amphiphilic properties of the copolymer. Furthermore, breakdown of the polymer is attributed to hydrolysis of the PLGA bonds and mPEG to form lactic acid, glycolic acid, and smaller fragments of mPEG.\textsuperscript{38–40} Breakdown of the copolymer can be slowed by increasing the ratio of LA to GA composition of the PLGA block.\textsuperscript{41,42} It has been reported earlier that release from such delivery systems is dependent on diffusion of the incorporated therapeutic and slow controlled breakdown of the copolymer.\textsuperscript{51} Consequently, by increasing the ratio of LA within the PLGA block, the hydrophobicity of the copolymer is increased, allowing decreased rate of copolymer breakdown which extends the release of the incorporated therapeutic. In addition, the weight to volume ratio at which the copolymer is mixed with water can also have an impact on polymer degradation and diffusion of therapeutics, and therefore the release of incorporated therapeutics. Because hydrolysis of the copolymer backbone is the key to polymer breakdown, having a higher copolymer content can avoid unnecessary or unwanted hydrolysis. However, with increasing copolymer content, the copolymeric solution becomes viscous, and consequently, the impact on solution injectability needs to be taken into consideration. With the help of electron microscopy, visual representation of the breakdown and surface deformities that occur as breakdown progresses can be obtained. Our previous work provides scanning electron microscopy images of the porous morphology, resulting from the breakdown of polymer and diffusion of molecules over the course of release.\textsuperscript{43}

![Figure 7.](image)

Table 1. (A) Secondary Structure Analysis of Salmon Calcitonin Released from Polymeric Formulations in Release Buffer at 37 °C and (B) Extracted from Polymeric Formulations Stored at 4 °C

| Sample Description                                      | Days | \(\alpha\) Helix | \(\beta\) Sheets | \(\beta\) Turns | Random Coils |
|---------------------------------------------------------|------|-----------------|-----------------|----------------|--------------|
| (A) sCT released from copolymeric formulations in release buffer at 37 °C |
| fresh sCT in PBS                                        | fresh sCT in PBS | 9.5             | 46.9            | 11.7           | 31.9         |
| sCT released in PBS                                     | 15   | 7.1             | 54.2            | 9.3            | 29.5         |
| sCT released in PBS                                     | 30   | 3.9             | 64.2            | 0              | 31.8         |
| (B) sCT extracted from copolymeric formulations stored at 4 °C |
| fresh sCT in PBS/ACN (1:1)                              | fresh sCT in PBS/ACN (1:1) | 16.2           | 35.6            | 3.9            | 44.3         |
| sCT extracted from copolymer depot                      | 15   | 14.9            | 40.7            | 1              | 43.5         |
| sCT extracted from copolymer depot                      | 30   | 13.4            | 41.6            | 1.7            | 43.4         |
volume contraction upon expulsion of the aqueous phase and push out effect. Second, the larger PLGA block gave insight into its role in slowing degradation of the copolymer, which in turn, slows release of therapeutic. Larger PLGA block makes the gel more hydrophobic making breakdown, which is primarily due to hydrolysis, more difficult. From this initial study, further testing using sCT was explored in this research using the insight gained. Both lysozyme and sCT retained bioactivity as evidenced by CD, MALDI-TOF mass spectrosopy, and HPLC analysis of entrapped and released therapeutic, demonstrating the ability of the copolymer to protect the structure of sensitive protein and peptide-based therapeutics. Release of therapeutics was observed over the course of 28 and 42 days for lysozyme and sCT, respectively. Burst release was minimized to ~22% for lysozyme and ~6% for sCT. The complete details and further insight into the rationale of the current work can be found in previous publication.

Furthermore, the aforementioned 3:1 thermosensitive copolymer was evaluated in vivo for sCT levels and serum calcium levels. The results demonstrated increased sCT level for over 40 days and decreased serum calcium levels for over a month. The therapeutic effect was further tested by investigating the ability to prevent methyldeniosolone acetate-induced osteopenia, and the results indicated retention of normal serum osteocalcin levels for up to 6 weeks using the depot formulation. On the basis of these data, a similar retention of bioactivity and therapeutic effect in vivo is expected using the formulation explored in the current study.

Others have found promising results in this area as well. In a recent study by Ding et al., a thermosensitive triblock copolymer of PLGA−PEG−PLGA was used as a controlled-release delivery system for a complex of sCT and oxidized calcium alginate. They found that in vivo in rats over 30 days that the treatment was effective in decreasing serum calcium and bone reconstruction while under glucocorticoid-induced osteopenia. In addition, Li et al. were able to investigate a supramolecular nanoparticle of a dipeptide (Asp−Phe, DF) that was complexed with sCT in vitro and in vivo. Release of over one month showed promise as a system that can bypass polymeric materials. This work supports the progress we are making in further development of controlled-release systems for osteopenia/osteoporosis.

The balance between hydrophobic and hydrophilic blocks in the copolymer is the driving force behind its transition from solution to gel. This delicate balance can be manipulated based on the structural composition of the copolymer. The hydrophobic effect in the presence of increased temperature drives the rearrangement of the hydrophobic and hydrophilic blocks in order to decrease entropy and be energetically favorable. The effects of altering the hydrophobicity of the polymer as a whole are evidenced by the sol−gel transition temperatures observed for each copolymer. In this study, sol to gel transition temperatures of mPEG−PLGA−mPEG copolymers of three different LA to GA ratios were tested using the tube inversion method and copolymer with LA/GA 5:1, where phase transition temperature <37 °C was found to be appropriate for further development into a controlled-release formulation. H1 NMR spectra confirmed successful synthesis of the mPEG−PLGA−mPEG copolymer with LA to GA ratio 5:1. Furthermore, GPC verified fairly uniform distribution of the purified copolymers evident by narrow and symmetrical distribution of retention peak and PDI relatively close to a value of 1. In general, a PDI value of less than 2 is considered an optimal polymerization method.

Copolymer mPEG−PLGA−mPEG forms micelles in aqueous solution, owing to their amphiphilic nature. The ability of copolymer mPEG−PLGA−mPEG to form micelles is yet another way in which the hydrophobic effect is evident. Amphipathic copolymer chains will rearrange in order to minimize interactions of hydrophobic blocks with the aqueous solvent. The hydrophobic domain induces assembly of the hydrophobic PLGA chains toward the core of the micellar structure and hydrophilic PEG chains facing the aqueous solvent. CMC is a unique characteristic concentration at which induction of micellar assembly takes place. CMC of the mPEG−PL5GA1−mPEG (LA/GA, 5:1) thermosensitive triblock copolymer was determined using a fluorimeter with pyrene as the hydrophobic fluorescence probe. Fluorescence of pyrene at increasing copolymer concentration was measured and intensity ratios of peaks at 379 and 393 nm were calculated. Once CMC was reached, a drastic decline in graph was seen because of decreased fluorescence detection of pyrene, owing to its entrapment within the micelles. Pyrene is attracted to the hydrophobic environment within the micelles and at CMC micellar assembly allows entrapment of hydrophobic pyrene, which can be seen as a sharp decline in intensity ratios of its first peak to the third. The point of sharp decline in fluorescence intensity in Figure 4 shows that the CMC of mPEG−PL5GA1−mPEG (LA/GA, 5:1) thermosensitive triblock copolymer is 25 μg/mL.

Initial research into thermosensitive delivery systems was limited because of toxicity caused by the use of organic solvents, such as with organogels and cytotoxicity of the polymers, such as poly(N-isopropyl acryl amide) and poloxamers (polyethylene oxide, polypropylene oxide) because of their inability to biodegrade. The development of mPEG−PLGA−mPEG triblock copolymers greatly improved thermosensitive polymer applicability, given their excellent biocompatibility and biodegradation. Trilob copolymer mPEG−PL5GA1−mPEG (LA/GA, 5:1) used in this study showed relative cell viability >80% for up to 1 mg/mL concentration with an IC50 > 10 mg/mL in HEK 293 cells. The products of polymer breakdown are lactic acid, glycolic acid, and smaller fragments of mPEG, which are naturally metabolized and excreted by the body and are therefore highly biocompatible. Furthermore, aqueous solubility of mPEG−PLGA−mPEG avoids the use of toxic organic solvents in the delivery system. It is not uncommon for controlled-release systems to exhibit burst release at or above 20% within the first 24 h. This has primarily been attributed to the amount of drug that lies near the surface of the gel implant and is readily released. However, when the concentration of the polymer and/or the hydrophobic block is altered to increase the overall hydrophobic nature of the polymer, diffusion can be reduced, and the burst release can be minimized. Eventually, breakdown of the polymer allows therapeutic molecules to be released, which consequently creates channels to be formed in the gel matrix, allowing for subsequent breakdown and diffusion to occur. The breakdown of the polymer can be tracked by measuring weight change over the course of release exposure to the aqueous environment as shown in our previous research, looking at the effects of molar mass and water solubility of incorporated molecules on the degradation profile of the triblock copolymer delivery system. In such instances,
the release profile may show a biphasic release or a drastically increased release rate toward the end of release. Release profiles of sCT demonstrated by copolymer mPEG−PLGA−mPEG (LA/GA, 5:1), used in this study at 30 and 40% (w/v), maintains steady release over the entire duration, justifying superior control of the copolymer over release of therapeutic on increasing the hydrophobic to hydrophilic block ratio when compared to other systems such as our previous work using only ∼3:1 LA to GA ratio copolymer. In addition, the increased w/v of the copolymer in our formulation from 30 to 40% also helps decrease burst release and provide for a longer duration of release. The constant supply of therapeutic will help maintain constant therapeutic level of sCT, thereby avoiding peaks and troughs concomitant to multiple administrations.

In recent years, several proteins and peptides have surfaced as an essential category of therapeutic drugs. However, their unique physiochemical and biological properties make them susceptible to chemical and physical degradation. Stability of protein therapeutics is one of the major challenges associated with controlled delivery of such drugs over a prolonged duration. Several enzymes and environmental factors pose challenges in vivo, necessitating frequent dosing of protein and peptide-based drugs. Thermosensitive copolymer depot-based controlled-drug delivery systems overcome stability challenges of protein-based therapeutics alongside providing a controlled release. These copolymers protect the native conformation of the sCT protein structure by masking it from the effect of surrounding environment in the depot form. Hence, the protein is maintained and released in its active conformation from such copolymeric depot-based delivery systems. CD spectroscopy confirmed the conformational stability of sCT released from the delivery systems in comparison to freshly prepared sCT solution. Storage stability of sCT incorporated in the thermosensitive copolymer stored at 4 °C also revealed that sCT maintains its conformational stability in comparison to freshly prepared sCT solution, owing to the protective effect of the copolymer incorporating sCT. The results indicate sCT is released from the mPEG−PLGA−mPEG (LA/GA, 5:1) copolymeric depot in its native conformation, which is essential for its biological activity. Our previous work with this polymer at a 3:1 LA to GA ratio further demonstrates the ability of the polymer to protect sCT as evidenced by CD, MALDI-TOF, and HPLC results. In addition, we know that sCT is relatively stable, but loss of native confirmation can reduce or completely eliminate its bioactivity. However, it has been shown that sCT undergoes denaturation only after prolonged exposure (>35 h) in aqueous solution at temperatures ≥40 °C, which is not expected under physiological conditions. Furthermore, PEG has been shown to prevent aggregation of sCT.

4. CONCLUSIONS

Patient compliance for effective and long-term management of chronic diseases such as osteoporosis is a major medical hurdle, and patients benefit most when therapeutic levels of drugs are maintained at an optimal concentration in the body without frequent administration. Controlled-release delivery systems, particularly subcutaneous depot systems help overcome this hurdle by making the dosing regimen easy, convenient, and consistent. To this end, we have developed and characterized a PEG−PLGA-based thermosensitive triblock copolymer for controlled delivery of sCT. The copolymer exhibited exceptional biocompatibility and demonstrated zero-order release profile of sCT over a period of ~60–70 days in a biologically active form. This thermosensitive copolymer-based delivery system could potentially deliver sCT at a controlled rate for up to two months following a single subcutaneous injection, thus improving patient compliance and quality of life in the treatment of osteoporosis. Further studies would entail determination of drug release in vivo, efficacy of the formulation in the treatment of osteoporosis in an appropriate animal model as well as long-term biocompatibility. Overall, ease of synthesis and incorporation of

![Scheme 1. Scheme of Synthesis of the mPEG−PLGA−mPEG Triblock Copolymer by Ring Opening Polymerization Reaction Followed by Diblock Condensation](image-url)
therapeutics in the thermosensitive copolymer-based controlled-delivery system used in this study can potentially change the conventional strategy for delivering various proteins and peptide-based therapeutic molecules.

5. MATERIALS AND METHODS

5.1. Materials. GA, MTT and isophorone disocyanate were purchased from Sigma-Aldrich (St. Louis, MO, USA). d,l-LA and methoxypolyethylene glycol-500 were acquired from TCI America (Portland, OR, USA) and Polysciences Inc. (Warrington, PA, USA), respectively. Stannous octoate was purchased from Pfaltz and Bauer Inc. (Waterbury, CT, USA). sCT was procured from Calbiochem (Burlington, MA, USA). Micro-bicinchoninic (micro-BCA) protein assay kit was obtained from Pierce Biotechnology Inc. (Rockford, IL, USA). Human embryonic kidney (HEK 293) cell lines, Dulbecco’s modified Eagle’s medium (DMEM), and PBS were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). All other chemicals were of analytical grade and used without further modification.

5.2. Synthesis and Characterization of Thermosensitive Triblock Copolymers. Thermosensitive triblock copolymer mPEG−PLGA−mPEG was synthesized using ring opening polymerization following diblock condensation (Scheme 1).

5.2.1. Sol to Gel Transition Temperature. Sol to gel transition temperature of the polymer with increasing LA/GA was determined using the tube-inversion method. Copolymer samples were dissolved in deionized water at 30 and 40% (w/v) concentration and injected into glass tubes immersed in a water bath was raised from room temperature (°C) while allowing the samples to acclimatize for 10 min at each temperature point. After 10 min, the glass tubes were inverted by addition of ice cold anhydrous toluene in a three-necked flask heated to 120 °C under continuous stirring. GA (2.32 g), LA (10.08, 12.96, or 14.4 g for 3.5:1, or 5:1, respectively) was dissolved in anhydrous toluene in a three-necked flask heated to 120 °C under continuous stirring. The copolymer sample was vacuum dried to completely remove residual organic solvents. The purified copolymer was vacuum dried to completely evaporate residual organic solvents.

5.2.2. 1H NMR Spectroscopy. The copolymer sample was dissolved in CDCl3 and analyzed using 1H NMR (Mercury Varian 400 MHz) spectroscopy to determine its structural composition. Tetramethylsilane was taken as the zero-chemical shift. Representative peaks for LA (−CH₃) and GA (−CH₂) components were integrated to determine LA to GA ratio of the copolymer. Bruker TopSpin 3.2.b.69 software provided with the NMR instrument was used for phase correction and evaluation of peaks.

5.2.3. Gel Permeation Chromatography. Copolymer sample (5 mg/mL) was prepared in tetrahydrofuran and analyzed using GPC (Tosoh Bioscience EcoSEC HLC-8320: modular system with refractive index and UV detectors) to find retention time and determine weight average molecular weight, number average molecular weight, and PDI of the synthesized copolymer.

5.2.4. Critical Micelle Concentration. CMC of the copolymer was determined using pyrene as the hydrophobic probe. Pyrene was dissolved in acetone (24 μg/mL) and 10 μL aliquots were added to each glass test tube. Acetone was evaporated, and 2 mL aqueous polymer solution was added to each tube at increasing polymer concentrations ranging from 0.5 to 1000 μg/mL. The test tubes were vortexed briefly multiple times followed by a resting period in the dark for 12 h. Fluorescence of pyrene at each concentration was measured using a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon, NJ, USA) with excitation set at 336 nm and emission from 360 to 450 nm (excitation and emission slit widths of 1 nm). Intensity ratios of peaks 379 and 393 nm were calculated and plotted against logarithm of concentration to determine CMC.

5.3. In Vitro Biocompatibility Assay. In vitro biocompatibility of the polymer was estimated using MTT cell viability assay using human embryonic kidney cell line (HEK-293) and DMEM. Cells were seeded in a 96 well plate at a density of 5 × 10⁴ cells per well followed by incubation at 37 °C and 5% CO₂. After 24 h of incubation, the media was replaced with fresh serum-free media containing increasing concentrations of the copolymer (0.1, 1, 2, 5, or 10 mg/mL). Cells were further incubated with the copolymer sample for 24 h. Cells incubated without any copolymer were taken as control. Following incubation, media was removed and 20 μL of MTT solution (5 mg/mL) was added to each well and incubated for 2 h to allow formation of formazan crystals. MTT solution was carefully aspirated and dimethyl sulfoxide (150 μL) was added to each well to dissolve formazan crystals. Absorbance was recorded at 570 nm, and relative cell viability was calculated using the following equation

\[
\text{Relative cell viability} (\%) = \frac{(A_{\text{polymer}} - A_{\text{DMEM only}})}{A_{\text{DMEM only}}} \times 100
\]

where, \(A_{\text{polymer}}\) is average absorbance of wells incubated with polymer samples and \(A_{\text{DMEM only}}\) is the average absorbance of the control wells incubated with serum-free DMEM.

5.4. In Vitro Release Profile of Salmon Calcitonin. Formulations were prepared by suspending sCT (2.5 mg) in 30 and 40% w/v aqueous copolymer solutions. Using a 25 G syringe, 0.5 mL of the formulation being tested was injected in each glass tube and incubated in a water bath at 37 °C to form a gel depot. Preserved PBS (10 mM, pH 7.4) containing 0.02% w/v sodium azide was then added to each tube as the release medium (4 mL per tube). The tubes were capped to prevent evaporation and incubated at 37 °C under constant shaking at 35 rpm. Sample aliquots (1 mL) were taken at selected time points and replaced with 1 mL fresh prewarmed release medium. Released protein sCT was quantified using micro BCA protein assay kit, following manufacturer’s instructions. Cumulative percent released was calculated over the course of delivery.

5.5. Stability of Salmon Calcitonin. Conformational changes in sCT released from the thermosensitive copolymer formulation were evaluated both at physiological and storage temperatures of 37 and 4 °C, respectively, using CD spectroscopy.

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5.5.1. Stability of Released Salmon Calcitonin at Physiological Temperature. Conformational stability of sCT released in vitro from thermosensitive copolymer formulation mPEG–PLA₃₅GA₉₅mPEG (LA/GA, 3.5:1) 40% (w/v) at 37 °C was analyzed at specific time intervals using circular CD spectroscopy. Samples were centrifuged, filtered, and degassed prior to analysis. PBS (10 mM, pH 7.4) was used as reference buffer. CD spectra were scanned in the far-UV region (190–230 nm) to investigate the changes in the secondary structure of sCT. All spectra were recorded at a scan rate of 50 nm/min at 20 °C using a quartz cuvette (0.1 cm path length). Freshly prepared sCT solution in PBS (10 mM, pH 7.4) was used as the standard. Spectra Manager2 software (Jasco, Tokyo, Japan) was used for spectrum analysis.

5.5.2. Stability of Salmon Calcitonin inside Gel during Storage at 4 °C. Conformational stability of sCT incorporated in thermosensitive copolymer formulation mPEG–PLA₃₅GA₉₅mPEG (LA/GA, 3.5:1) 40% (w/v) at storage temperature (4 °C) was also determined using CD spectroscopy as aforementioned. sCT was extracted from these formulations using ACN/PBS (1:1, v/v) at predetermined time points. The stability of sCT, as evidenced by the presence of two peaks in ACN/PBS, was comparable with that of freshly prepared sCT [1 mg/mL, ACN/PBS (1:1, v/v)].

5.6. Data Analysis. All data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using tailed Student’s t-test and ANOVA. A p value of less than 0.05 was considered to be significant.

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ABBREVIATIONS
LA, δ-l-lactide; GA, glycolide; mPEG, methoxypolyethylene glycol-500; sCT, salmon calcitonin; 1H NMR, proton nuclear magnetic resonance; GPC, gel permeation chromatography; CMC, critical micelle concentration; HEK 293, human embryonic kidney cell line; Mₙ, number average molecular weight; Mₚ, weight average molecular weight; PDI, polydispersity index; PBS, phosphate buffer saline; MTW, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; DMEM, Dulbecco’s modified Eagle’s medium

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