In Vitro Evaluation of PLGA-PEG-PLGA Microspheres for Sustained Release of Insulin

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Abstract : Formulating a polypeptide gastrointestinal drug delivery system have been persistent challenges. To overcome thesechallenges, microsphere are more attractive because of their biodegradability and easy preparation and administration. In this study, biodegradable triblockcopolymerpolylactide-co-glycolide- polyethylene glycol-poly lactide-co-glycolide (PLGA-PEG-PLGA) was synthesized under microwave for insulin encapsulation and release. PLGA-PEG-PLGA was characterized by ^{1}HNMR and gel permeation chromatography. The sol–gel transition temperatureof PLGA-PEG-PLGA was also evaluated using refrigerated bath circulator instrument. The solution of PLGA-PEG-PLGA and insulin was injected in distilled water (45 ^{o}C) in probe sonication system during 1 min for achieving the microsphere without using any organic solvent. The amount of insulin encapsulated in the PLGA-PEG-PLGA microspheres were determined by the Bradford method. Microspheres of an average particle size of 34 ± 8.3 µm that were observed using optical microscope were used for an in vitro release study. Finally, CD spectrum tests were performed to approve the stability of released insulin. It was demonstrated that the synthesis of PLGA-PEG-PLGA via microwave irradiation was fast and efficient. In vitro release studies affirmed the sustained release profile of insulin. Results of stability tests confirmed the stability of insulin following release.
Key Words : PLGA-PEG-PLGA, Insulin, Microsphere, Sustained release.

Introduction

Insulin is a protein of immense importance because of its role in the treatment of diabetes, which is growing in epidemic proportions in many developing countries[1]. Insulin-dependent diabetes mellitus patients usually have to boost the basal insulin supply once or twice a day, in addition to doses at mealtimes, because they need a relatively constant basal level of insulin to achieve a near physiological pattern of insulin secretion. Such therapy has been reported not only to improve their general condition, but also to reduce the incidence of diabetic complications. If an insulin formulation was available that could release the drug in a constant level for longer periods, patients would be freed from the need to administer multiple doses[2, 3].

Different systems based on biodegradable polymers have been explored for these purposes. Special attention was placed in homo and copolymers derived from lactic acid (i.e. D and L-poly(lactic acid), poly

(lactic-co-glycolic acid), poly lactide-co-glycolide- polyethylene glycol-poly lactide-co-glycolide (PLGA-PEG-PLGA), and etc. Several devices, such as microspheres, nanoparticles, hydrogels, lipid-based systems, *in situ* prepared parenteral drug delivery systems and complexes have been investigated to entrap and release a great number of drugs [4].

PLGA-PEG-PLGA is a tri-block copolymer that consists of polyethylene glycol (PEG) and two polylactideglycolide (PLGA) blocks; the latter are composed of lactide (LA) and glycolide (GA) monomers. PLGA is a hydrophobic block due to the presence of LA, while PEGis a hydrophilic block. Zentner and coworkers were the first to synthesize this copolymer (Regel) and to demonstrate its thermoresponsiveness and in situ forming properties [5]. The copolymer is biocompatible and biodegradable; therefore, it is a good candidate as a drug delivery system[6].

Different therapeutic cargos such as DNA molecules, peptides (growth hormone[7], venom peptide [8]), proteins (insulin [9]), and other drug molecules (paclitaxol[5], naltrexone hydrochloride[10, 11]) have been loaded into this hydrogel and both in vitro and in vivo investigation have demonstrated the suitability of PLGA-PEG-PLGA hydrogel for therapeutic applications. Various parameters such as PEG molecular weight (Mw), LA to GA ratio (LA:GA), tri-block copolymer Mw, copolymer concentration, and persistence of other molecules in the copolymer formulation (such as drugs and salts) can be manipulated to alter the properties of the system, such as its phase transition temperature, viscosity, swelling, degradation rate, and drug release profile [12-14].

For the first time, Kwon and Kim [15] encapsulated zinc crystalline recombinant insulin within biodegradable triblock PLGA-PEG-PLGA microspheres in aqueous medium based solely on the thermosensitive sol–gel transitionproperty of the aqueous solution of triblockand mineral oil without using an organic solvent. But in the end of process, Hexane as an organic solvent was used for purification of microspheres.

In the present study we describe microsphere preparation from a biodegradable triblock copolymer, without using of any organic solvent even in the purification stage, utilizing its thermosensitivity property of PLGA-PEG-PLGA copolymer in aqueous medium. We studied in vitro release of insulin from microsphere. The secondary structure of insulin released was investigated by far-UV circular dichroism (CD) spectroscopy.

Materials and Methods

Reagents and solutions

Polyethylene glycol (PEG) with a molecular weight of 1000 was purchased from Merck (Germany). Glycolide A, D,L-lactide, and stannous 2-ethylhexanoate were purchased from Sigma Aldrich, USA.Insulin were kindly donated by the Novo Nordisk representative in Tehran, Iran. All other analytical grade chemical reagents and solvents were obtained from Merck (Germany) and used as received.

Synthesis and Purification of PLGA-PEG-PLGA

PLGA–PEG–PLGA with a lactide (LA)-to-glycolide (GA) ratio of 3:1 was synthesized using a ring-opening method with microwave irradiation. Briefly, polyethylene glycol (PEG, MW= 1000, 6 g) was introduced into a round-bottom flask equipped with a condenser and then placed in a microwave (Milestone, Italy). While the samples were being stirred at 200 rpm, they were irradiated at 800 w for 10 min at 120 °C. Next, 11.35 g of D,L-lactide and 3.48 g of glycolide were added, and the mixture was heated and stirred at 150 °C under a vacuum at 800 w for 5 min. As a catalyst, 0.04 g of stannous 2-ethylhexanoate was added, and the heating and stirring was continued at 150 ± 1 °C under a vacuum at 800 w for 5 min. The synthesized copolymer was purified by dispersing it in 30 mL of water (40 °C) and the temperature of the dispersion was subsequently reduced to 4 ± 1 °C to completely dissolve the copolymer in water. By heating the solution to 60 °C, the triblock copolymer was precipitated, and all of the impurities remained in the solution. The purification process was repeated 3 times, and the precipitates were freeze-dried and kept at -20°C until use[5, 16].

Characterization of Tri-block copolymer

The copolymer structure and number-average molecular weights (M_n) were determined using Nuclear Magnetic Resonance Analysis (^{1}HNMR), (Brucker, Germany) at 400 MHZ and 25°C in CDCl$_3$[17].Gel

permeation chromatography (GPC) (RID-A refractive index signal detector) was utilized to determine the weight-average molecular weight (M_w), M_n, and polydispersity of the block copolymers using tetrahydrofuran as the solvent and eluent at a rate of 1mL/min and polystyrene as the calibration standard. The sol–gel transition temperature was determined by a refrigerated bath circulator instrument (WISD P-22, South Korea). PLGA–PEG–PLGA copolymers were dissolved in phosphate-buffered solution (PBS, pH 7.4) to make the concentrations of 17, 23, and 28 wt%. The temperature was then increased by 0.5 $^{\circ}$C/min, from 0 $^{\circ}$C to the temperature at which the magnet inside the copolymer solution stopped stirring and the gel formed[14].

Preparation and characterization of PLGA-PEG-PLGA microspheres encapsulated with insulin

First, an aqueous solution of insulin (0.018 mg/mL) in HCl 0.1N was prepared and NaOH 1N was added up to pH 7.4 and the temperature decreased to 4^{0}C. Then, 0.5 mL of PLGA-PEG-PLGA solution was added to 0.5 mL of the 0.018 mg/mL insulin solution to reach to final concentration of 15% w/w copolymer, and was stored at 4 $^{\circ}$C for preserving secondary structure of insulin. After that, the solution of PLGA-PEG-PLGA and insulin was injected in distilled water (40 $^{\circ}$C) in probe sonication system during 1 min for achieving the microsphere. The microspheres were collected by centrifugation at 19,000 rpm at 40°Cfor 15 min, followed by dispersion in 5 mL of distilled water (38 $^{\circ}$C). Mannitol (1% w/v, 5 mL) was added to the resultant microspheres suspension, flash-frozen with a dry ice–acetone mixture, and freeze-dried. The microsphere powders were kept at −20°C for further use. To determine the measurement of microsphere size, 20 mg of freeze-dried microspheres were added in 1 mL of distilled water at 40°C, and then was stirred. After that, microsphere sizes were observed using optical microscope with lenses of 10x. All measurements were performed in triplicate at 25°C.

In Vitro Drug Release Study from PLGA-PEG-PLGA microspheres

Phosphate buffer pH 7.4 (600 µl) was added to each microtube containing 30 mg of microspheres (blank or insulin loaded). The microtubes were submerged in a reciprocal water bath (NBIOTEK NB -304, South Korea) (20 rpm) at 37^{0}C. At specified time intervals, a 500-µl aliquot was withdrawn from each sample and replaced with 500 µl of fresh PBS. The samples were assayed for insulin content by Bradford assay. Each sample with Bradford reagent (n=3) was analyzed in a 96-well plate by spectrophotometry measurement at 595 nm using an ELISA microplate Reader (BioTeck, USA).

In each microplate, the 3 first wells of the 3 first rows (Wells A) were considered as blank. Wells B to G were filled with standard insulin in amounts of 2.5, 5, 10, 15, 20 and 25 µl, respectively. Then filled up to 25 µl by PBS (pH=7.4). Other wells were filled with 25 µl of samples from each day, respectively (n=3). Into each well, 250 µl fresh protein reagents was added, and after 5 minutes, the test was carried out.

Preparation of protein reagent

Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 mL of 95% ethanol. To this solution, 100 mL of 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 L. Final concentrations in the reagent were 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid [18]. The reagent was filtered through Whatman No. 1 filter paper and then stored in an amber bottle at room temperature. It can remain stable for several weeks, however during this time dye may precipitate from the solution, and so the stored reagent was filtered before use [19].

Preparation of standard protein

Bovine γ-globulin at a concentration of 1 mg/ml (100 µg/ml for the micro assay) in distilled water is used as a stock solution. This should be stored frozen at -20ºC[19]. In the current study, insulin (100 µg/ml in PBS) was used as stock solution.

Far-UV Circular Dichroism (CD) Spectroscopy

The secondary structure of insulin released from hydrogels after the in vitro release experiment was investigated by CD spectroscopy, Jasco J-815. The samples were first filtered through a 0.2 µm syringe filter to separate the protein from the polymer. As the control, CD spectra of dissolved insulin in the extracting medium were collected and measured at room temperature using a J-815 spectropolarimeter from Jasco (Japan) with a 1

mm light path quartz cell (Hellma, Müllheim, Germany). The conditions of analysis were as follows: cell length, 1 cm; scanning speed, 100 nm/min; data pitch, 0.1 nm; sensitivity, standard; band width, 1 nm; wavelength range, 190–250 nm (far UV). The samples and standard insulin solution were prepared in 25 mMTris-HCl solution (pH=7.8). The CD pattern was compared with the CD pattern of standard insulin[3].

Data analysis

The results were reported as means ± SD (n ≥ 3). Data were analyzed by one-way analysis of variance (ANOVA). A probability value of less than 0.05 was considered significant.

Results and Discussion

The microwave heating process, the high temperatures attained, and the ability to work under high pressure conditions in a relatively short time make reactions faster and limit the occurrence of slower side reactions; thus, greater yields are usually obtained [20].Owing to a number of unique advantages, including shorter reaction time, higher yield, limited generation of by-products, and the relatively easy scale-up procedure with no detrimental effects, this technology has quickly become an appealing synthetic tool. As shown in figure 1, the ^{1}HNMR results are similar to those previously reported by Khodaverdi et al.[21].The LA-to-GA was determined to be 2.9 by ^{1}HNMR, which matched the initial ratio of monomers used in the copolymerization. The spectrum was similar to that reported by Chen *et al.*[22], and it verified the correct synthesis of the triblockcopolymer. The number average molecular weight (M_n), the weight average molecular weight (M_w), and molecular weight distribution of the copolymer were determined by GPC and ^{1}HNMR (table 1).The GPC chromatogram of the synthesized copolymer is shown in figure 2. The GPC curve of the copolymer is symmetric and unimodal. No shoulder peaks corresponding to the lactide and/or glycolidemacromers or their homopolymers were detected. The ratio of copolymer Mw determined by GPC and M_n estimated by ^{1}HNMR was 1.22, which confirms the effective synthesis of the copolymer with low polydispersity. The results of polymer characterization tests verified that microwave irradiation is a reliable method for the synthesis of PLGA-PEG-PLGA triblockcopolymers. Figure 3 shows the gelation diagram of the triblock copolymer (17%, 23%, 28%) aqueous solutions. The transition temperature decreased with increasing copolymer concentration, from 17 to 28 wt%. The sol-to-gel transition of an aqueous solution of the triblock copolymer is probably due to micellar packing, which is promoted by an increase in aggregation number driven by hydrophobic forces and the weakness of the hydrogen bonding with water molecules[10]. Preparation of aqueous-based microspheres was based solely on the thermosensitive sol–gel transition property of the aqueous solution of PLGA-PEG-PLGA. The amount of insulin encapsulated in the PLGA-PEG-PLGA microspheres that determined by the Bradford method was 54.3 %. Microspheres of an average particle size of 34±8.3 µm that were observed using optical microscope fitted with calibrated reticle were used for an in vitro release study.

The feasibility of using PLGA-PEG-PLGA microspheres was investigated in order to achieve the goal of controlled release of insulin. As can be seen in figure 4, in vitro release exhibited sustained and nearly incomplete release of insulin (42 %) over 3 weeks. The first phase of insulin release (first 18 days) from microsphere seems to be dependent more on diffusion. Then after day 18 the insulin release rate turned to an increasing mode, and probably degradation of the matrix, at this time point, begins to play a more significant role in release than in the earlier phase. The release kinetics of insulin showed the best fit for the Higuchi model ($R^2 = 0.9026$, Eq.1), followed by zero-order release kinetics ($R^2 = 0.9923$, Eq.2) (table 2).

$$Q=2C\sqrt{Dt/\pi} \qquad\qquad Q\ \alpha\sqrt{t} \qquad (3)$$
$$Q=KtQ\alpha t \qquad (4)$$

Where t is time, D is the diffusion constant, C is the initial drug concentration, K is the constant, and Q is the mass flux[10].

Since insulin may lose its activity during formulation, it is important for the controlled delivery system to release insulin in its active form. CD analysis demonstrated that the secondary structural integrity of insulin was maintained during preparation and release processes. CD spectra (figure 5) showed that the conformation of released insulin from microsphere was unchanged after 20 days; insulin was released in its native state and was preserved its secondary structural integrity,

Conclusion

In the current study, copolymers were synthesized under microwave irradiation. This technique appeared to be fast and efficient. Aqueous-based microspheres of low-MW PLGA-PEG-PLGA, a thermosensitive and biodegradable triblock copolymer, were prepared by utilizing its sol–gel transition property without using organic solvent even in the purification stage. In vitro release exhibited sustained and nearly incomplete release of insulin over 3 weeks. CD analysis demonstrated that the secondary structural integrity of insulin was maintained during preparation and release processes.

Acknowledgment

Table 1 Comparisons of ^{1}HNMR and GPC data for PLGA-PEG-PLGA copolymer.

^{1}H-NMR			GPC		
M_n^a	LA/GA[b]		M_n^c	M_w^d	M_w/M_n^e
1116-1000-1116	2.90		3290.3	4734.7	1.22

[a] Average molecular weight determined by ^{1}HNMR
[b] LA/GA ratio determined by ^{1}HNMR
[c] Number average molecular weight determined by GPC
[d] Weight average molecular weight determined by GPC
[e] Polydispersity determined by GPC

Table 2 Kinetics release of insulin from PLGA-PEG-PLGA microsphere

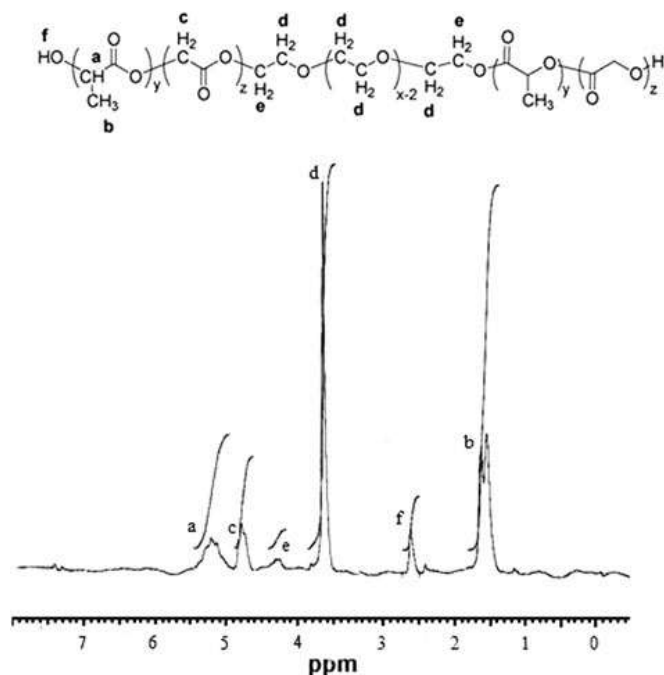| Drug (0.018 mg/mL)	Copolymer (0.88 mg/mL)	Zero-order equation			Higuchi equation	
		Slope	R^2		Slope	R^2
Insulin	PLGA-PEG-PLGA	2.3658	0.9923		2.9958	0.9026

Figure 1: The ^{1}H NMR spectrum and chemical structure of the PLGA–PEG–PLGA copolymer.
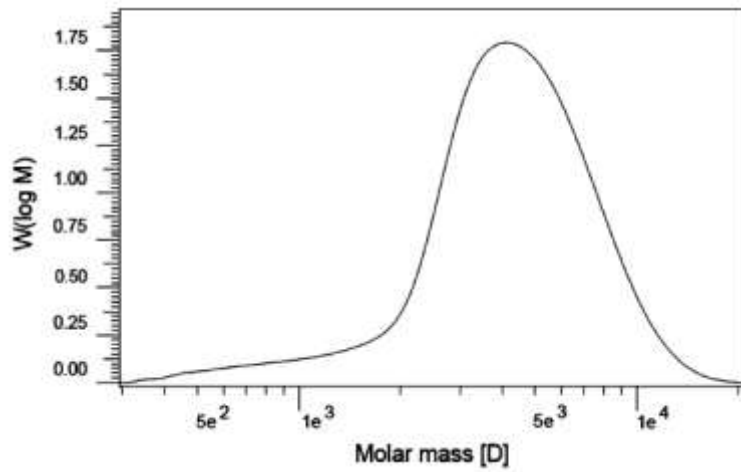
Figure legends

Figure 2: A GPC chromatogram of the PLGA–PEG–PLGA copolymer.
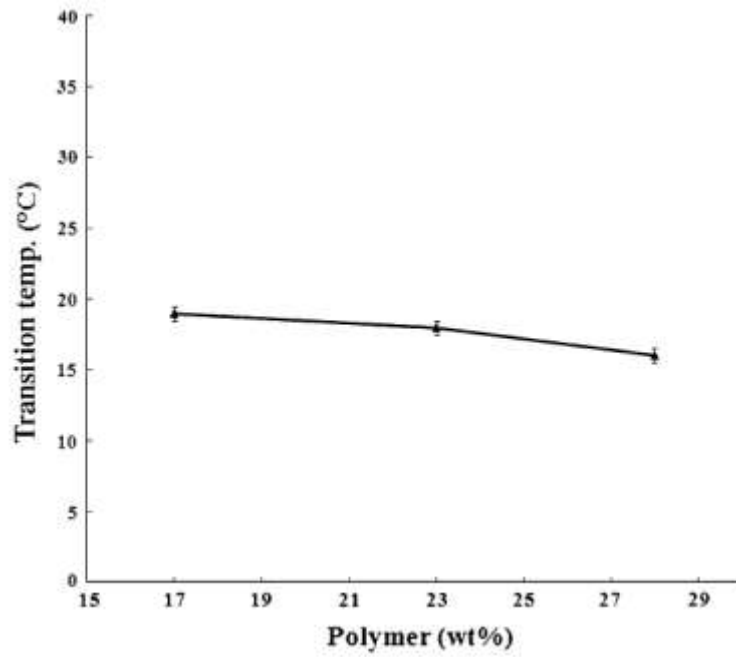

Figure 3: The phase diagram of the PLGA–PEG–PLGA copolymers in aqueous solutions with different polymer concentrations.
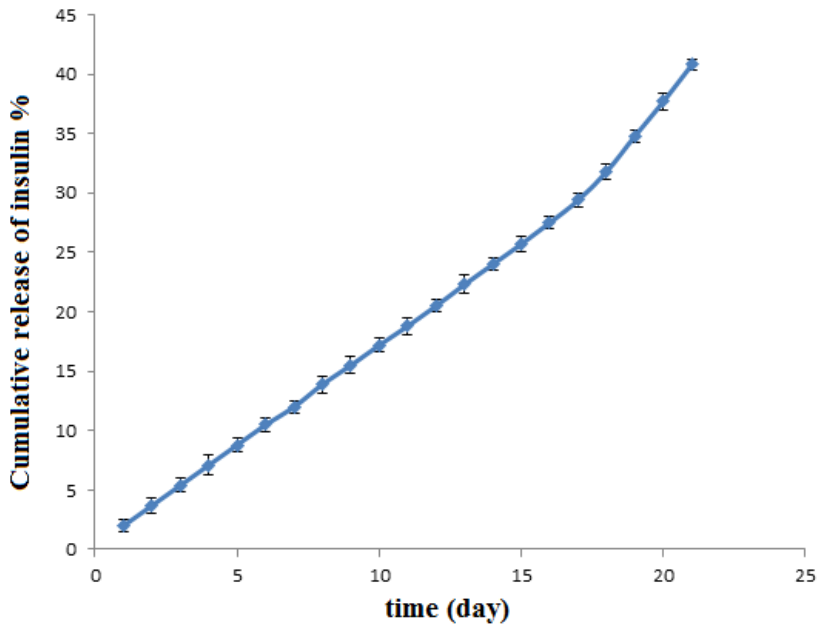
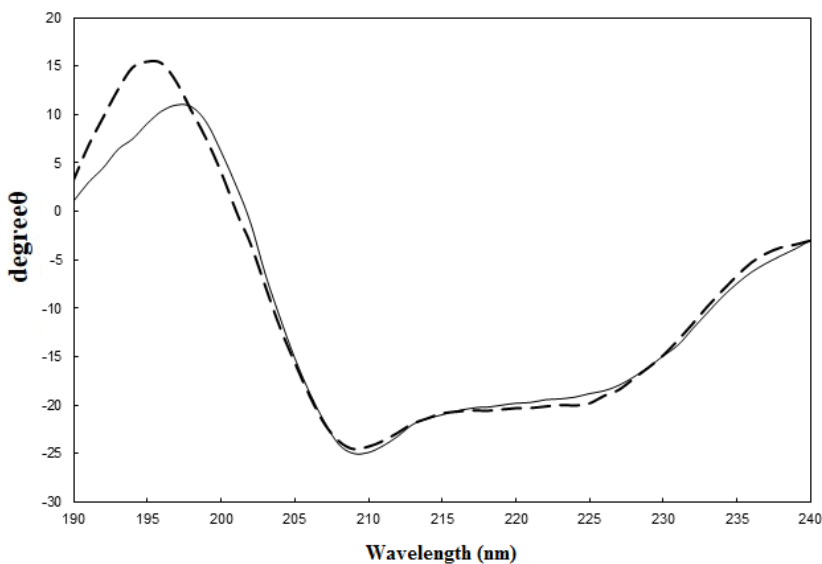**Figure 4:Drug release profiles of microsphere during 21 days.**

Figure 5: CD spectra of native insulin and insulin released from microsphere. ----- **shows insulin released from microsphere and** ——— **shows native insulin**

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