# Biodegradable Triblock Copolymer Microspheres Based On Thermosensitive Sol-Gel Transition

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***Purpose.*** The purpose of this study is to design microspheres for sustained protein delivery using thermosensitive, biodegradable triblock copolymer, poly (D,L-lactide-co-glycolide)-b-poly (ethylene glycol)-b-poly (D,L-lactide-co-glycolide) (PLGA-PEG-PLGA) without using organic solvent.

***Methods.*** Microspheres of the triblock copolymer PLGA-PEG-PLGA were prepared in an aqueous-based method without using methylene chloride (Msp A). This method used the sol–gel transition property of the polymer. The size and morphology of the microspheres were examined by optical microscopy and scanning electron microscopy (SEM). Zinc crystalline recombinant human insulin was incorporated in Msp A as well as in the microspheres of the same polymer prepared by the conventional water-in-oil-in-water (w/o/w) double emulsion method using methylene chloride (Msp B). Insulin release from both microspheres was carried out using high-performance liquid chromatography (HPLC) as well as circular dichroism (CD) spectroscopy of released insulin. FITC-insulin-loaded Msp A and Msp B were observed under confocal microscopy. Both microspheres were injected subcutaneously to SD rats with diabetes induced by streptozotocin. Blood glucose and plasma insulin levels were monitored.

***Results.*** Although the insulin release from Msp B exhibited initial burst and incomplete release, Msp A showed significant reduction of initial burst and continuous release over 3 weeks (>85%). CD spectra of released insulin showed that insulin from Msp A preserved its secondary structural integrity, whereas that from Msp B indicated changes in conformation. Confocal microscopy of FITC-insulin-loaded microspheres (both A and B) showed that the observed release profile may be attributed to homogeneous distribution of FITC-insulin within Msp A but inhomogeneiety in Msp B. Both microspheres were injected s.c. to diabetic rats. Whereas Msp B caused a burst effect (hypoglycemia) followed by quick change in blood glucose and insulin level, Msp A exhibited relatively sustained release of insulin and blood glucose level for at least 10 days.

***Conclusions.*** The PLGA-PEG-PLGA microspheres (Msp A) demonstrated continuous release of insulin *in vitro* and *in vivo* without serious burst effect and incomplete release, as shown by Msp B.

**KEY WORDS:** biodegradable triblock copolymer; microspheres; insulin release; thermosensitive polymer.

## INTRODUCTION

As a number of therapeutic protein drugs became available, the quest for suitable release systems for protein drugs using biodegradable polymers has been an active area of research (1–9). Most of the protein drugs have to be administered by frequent injections that hinder their widespread use. Thus, research effort has been concentrated on sustained-release delivery systems. By far the most popular approach to develop an injectable sustained-release system is to entrap the protein drug into microspheres prepared from biodegradable polymers such as homo- or copolymers of lactic and glycolic acids (PLGA) (1–9), which are well tolerated because they are nontoxic and nonimmunogenic.

Examples of successful delivery system for protein or peptide drugs include Lupron Depot for the delivery of the LH-RH agonist leuprolide acetate for 1 or 3 months (1) or Nutropin Depot for sustained delivery of human growth hormone for 1 month (4). Protein-loaded microspheres are prepared via either a w/o/w double-emulsion solvent evaporation method or a spray-dry method, which usually involves the use of a water-immiscible, volatile organic solvent such as methylene chloride ($CH_2Cl_2$).

However, unresolved drawbacks associated with protein-loaded microspheres in the context of protein release and stability still exist. Initial burst release followed by slow and incomplete release of proteins has been frequently observed (2). The burst effects could result in serious side effects, whereas incomplete release means a less-than-effective plasma concentration of therapeutic agents. In addition, a residual level of organic solvent that is difficult to remove completely may bring about toxicity issues. The use of an organic solvent as well as the harsh preparation conditions are thought to be main reasons for these observed drawbacks that hamper controlled delivery of protein drugs. In addition, physical degradation of proteins can occur at the interface of the aqueous phase and the organic phase (6,8).

An alternative approach for an injectable system for controlled release of protein drugs is the use of thermosensitive polymers that exhibit lower critical solution temperature (LCST) in water, such as poly (N-isopropyl acrylamide) (PNIPAAm) (8), or polymers that are able to form a hydrogel around body temperature. These are capable of entrapping drugs on injection into the body. Examples are low-molecular-weight triblock copolymers such as poly(ethylene glycol; PEG)-b-poly(propylene glycol)-b-PEG (poloxamers) (10), and more recently, PEG-PLGA-PEG (11–15) or PLGA-PEG-PLGA (16,18) are hydrophilic/hydrophobic balanced polymers that exhibit thermosensitive sol–gel transitions above certain critical solution temperatures. The latter two are of especial interest in developing injectable drug delivery systems because of their biodegradability without tissue irritation (13,18).

In this study we describe microsphere preparation from a biodegradable triblock copolymer, without using toxic, water-immiscible organic solvents such as $CH_2Cl_2$, utilizing its thermosensitivity as a mode of microsphere formation. We studied insulin release, *in vitro* and *in vivo*, using aqueous-based microsphere (A) prepared without using $CH_2Cl_2$ in contrast to the microsphere (B) of the same polymer prepared from the double emulsion method using $CH_2Cl_2$.

## EXPERIMENTAL METHODS

### Materials

PLGA-PEG-PLGA (MW ~4000, 1500-1000-1500 by $^1$H-NMR, Tg ~ −3° to −4°C), which was synthesized via ring-

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opening polymerization of D,L-lactide and glycolide (75:25 mol:mol) by PEG diol (MW ~1000) in the presence of stannous octoate as catalyst (16,18), was kindly provided by Macromed, Inc. (Sandy, UT). An aqueous solution of PLGA-PEG-PLGA was prepared at 4°C. The solution (23–28%, w/w) exhibited sol–gel transition at 10–15°C, above which the solution viscosity steeply increased to form a stable hydrogel (18). Recombinant human insulin (Pentex®, Zn salt, Lot No. 506, 27 IU/mg) was purchased from Beyer. Fluorescein isothiocyanate (FITC)-labeled insulin was purchased from Sigma (St. Louis, MO). All other chemicals were of reagent grade and used without further purification.

Insulin was crystallized in the presence of 7% NaCl and 0.1 M sodium acetate. Zinc chloride was added so that there are about four to five zinc atoms per insulin hexamer (19,20). Then, pH was adjusted to 5.5 and the solution incubated at 4°C for 2 days.

### Microsphere Preparation

*Aqueous-Based Microsphere (Msp A)*

Aqueous solution of PLGA-PEG-PLGA (28% w/w) was prepared at 4°C in 20 mM Tris buffer, and pH was adjusted to 7.4. Crystallized Zn-insulin suspension was added to the solution. An aliquot of 0.5 ml polymer solution was placed in 50 ml of mineral oil at 4°C and stirred in a baffled cell at for 15 min at various stirring rates (300–1000 rpm). The resultant mixture was transferred to a 4" × 4" weighing dish, and temperature was raised to 35°C and maintained for 10 min. Microspheres were formed on the surface of the dish and washed with hexane followed by 10 mM Tris buffer. Mild conditions were used to prepare microspheres to guarantee prevention of microsphere aggregation and protein denaturation with high yield. Microspheres were collected in a vial for *in vitro* release.

*Microsphere Prepared by w/o/w Double-Emulsion Method (Msp B)*

The triblock copolymer was dissolved in methylene chloride (1 g in 3 ml $CH_2Cl_2$). Zn-insulin powder was dispersed in 1 ml of Tris buffer of pH 7.4 and homogenized into polymer solution in $CH_2Cl_2$ for 1 min. This mixture was poured into a 150 ml of mildly stirred Tris buffer [1% poly(vinyl alcohol)] at 35°C. The spherical organic phase turned into microspheres as the solvent diffused into the buffer and evaporated. Microspheres were collected after 2 h for release study.

In both Msp A and Msp B, the same condition was used (Zn-insulin and the same triblock copolymer) except for the solvent and preparation method. Loading content was determined by dissolving the microspheres in cold water/acetonitrile mixture (1:3 v/v) followed by HPLC analysis under the same condition for *in vitro* release study, as will be described. Loading, entrapment efficiency, and microsphere yield for both Msp A and Msp B are summarized in Table I.

**Table I.** Summary of Insulin-Loaded PLGA-PEG-PLGA Microspheres

| Microsphere | Yield (%) | Loading efficiency (%) | Loading (%) |
|---|---|---|---|
| MspA | 91 | 87 | 3.5 |
| Msp B | 73 | 62 | 4.2 |

### Size and Morphology

Size distribution before lyophilization was determined using a Nikon optical microscope fitted with a calibrated reticle. The average diameters of microspheres were determined at different stirring rates. Surface morphology of freeze-dried microspheres loaded with insulin was observed via scanning electron microscopy (Hitachi 3500). Microspheres were lyophilized in the presence of D-mannitol and kept in a freezer at −18° to −20°C (below the Tg of the polymer) throughout the freeze-drying process for 5 days. Freeze-dried microspheres redispersed in aqueous medium at 37°C were also observed. Confocal microscopy (Zeiss LSM-510) was performed for both types of microspheres (Msp A and Msp B) in which FITC-insulin is incorporated.

### Insulin Release *In Vitro*

Microsphere-containing vials (0.1 g of microspheres, n = 3) were incubated in a shaker water bath at 37°C, 30 strokes/min. Microspheres of average particle size of approximately 100 μm were used for *in vitro* release. For each sample, release medium (10 mM Tris buffer at pH 7.4, 0.14 M NaCl) was changed at every 24-h interval. Amount of released insulin was determined by reverse-phase high-performance liquid chromatography (RP-HPLC) with binary gradient elution [water and acetonitrile with 0.1% (v/v) trifluroacetic acid (TFA)] (17). Circular dichroism (CD) spectra were taken for released insulin on Jasco-720 spectropolarimeter.

### Insulin Release *In Vivo*

Animal experiments were carried out in accordance with the "Principles of Laboratory Animal Care." Streptozotocin (STZ) in citrate buffer (pH 4.5) was given by intraperitonial injection to male Sprague-Dawley (SD) rats (55 mg/kg) to induce hyperglycemia. Both Msp A and Msp B (lyophilized) were dispersed in 2% carboxymethyl cellulose containing 5% D-mannitol as an injection vehicle (1). Microspheres were injected subcutaneously to diabetic rats (260 ± 10 U/kg rat). Aliquots of blood were drawn from tail veins once a day, except the first day. Plasma insulin level was determined using a radioimmunoassay (RIA) kit (ICN Pharmaceuticals), and blood glucose level was also monitored using AccuCheck® (16). Five rats were used for each group, and the diabetic rats with no injection were considered as control.

## RESULTS AND DISCUSSION

The triblock copolymer, PLGA-PEG-PLGA, has been demonstrated to serve as an aqueous-based nontoxic biodegradable drug release system for protein and water-soluble drugs (16,18) because of its thermosensitive property and degradability in water. At 37°C, this polymer degrades in approximately 6 weeks *in vitro*. Degradation products include lactic acid, glycolic acid, and PEG 1000 diol. The *in vivo* degradation is reported to occur in 4 weeks in rats.

Preparation of aqueous-based microspheres (Msp A) was based solely on the thermosensitive sol–gel transition property of the aqueous solution of PLGA-PEG-PLGA. Size distribution of microspheres was evaluated by using an optical microscope fitted with calibrated reticle. The average size of

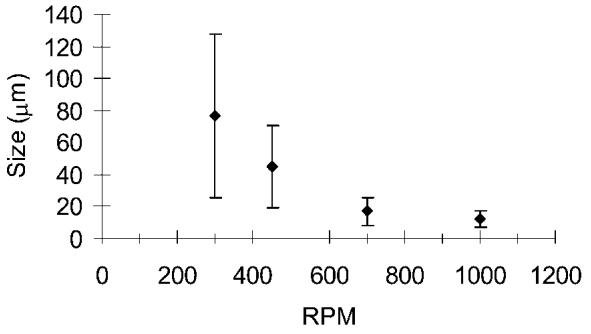


**Fig. 1.** Average diameter of Msp A as a function of stirring rate in the baffled cell.

the microspheres can be varied by using different stirring rates in the baffled cell. As shown in Fig. 1, the mean diameter of the PLGA-PEG-PLGA microspheres can be seen as a function of stirring rate. For example, microspheres prepared at the stirring rate at 700 rpm yielded a mean size of 17 ± 9 μm (mean ± S.D.; n = 200).

The microspheres were lyophilized for observation of surface morphology. Because the Tg of the polymer is low (below zero), freeze-drying should occur well below Tg. The SEM images of a freeze-dried microsphere are shown in Fig. 2. Spherical morphology was maintained after lyophilization, as well as the display of smooth surface. Mannitol was used as a lyoprotectant. Also, in a separate test, the freeze-dried, cold microspheres can be reconstituted in aqueous media at 37°C with no significant aggregation (data not shown). However, transfer must be done quickly so that the freeze-dried microspheres would not undergo glass transition before redispersion.

*In vitro* release studies were carried out with both Msp A and Msp B. As can be seen in Fig. 3, Msp A exhibited sustained and nearly complete release of insulin over 3 weeks. The first phase of insulin release (first 10 days) from Msp A seems to be dependent more on diffusion because the release rate was decreasing slightly. Then after day 10 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. Msp A was prepared in a milder environment than the w/o/w method in that organic solvent and high shear were absent.

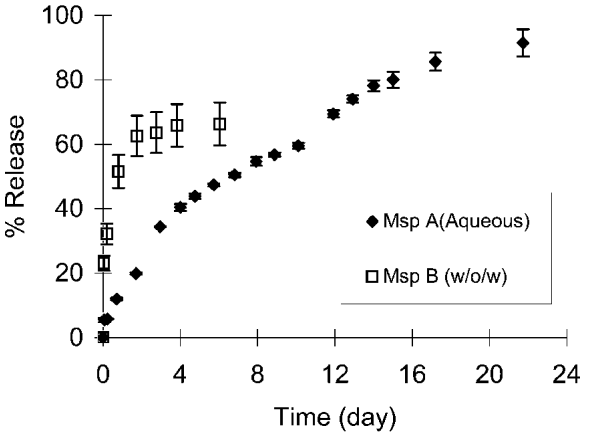


**Fig. 3.** *In vitro* release of human insulin from Msp A (aqueous-based) and Msp B (methylene chloride-based) (n = 3, mean ± S.D.)

Also, insulin was crystallized in the presence of high chloride concentration and acetate as well as zinc. Zinc content is known to be critical (~4–5 atoms per hexamer) in producing crystal suspension. These crystals exhibited rhombohedral shape under the optical microscope (19). In the presence of extra Zn, insulin can be more stable over a prolonged period of time (20).

Msp B exhibited initial burst release (~50% in 1 day), and release was stopped at ~60% after that. In preparing Msp B, during the formation of primary emulsion where it involves high shear and heat generation to create a large water/organic solvent interfacial area, proteins, especially when dissolved, can undergo rapid aggregation under this environment (8) and thus the incomplete release of proteins from PLGA microspheres may be caused by these trapped aggregates formed during microsphere fabrication. This accounts for slow and incomplete release after the initial release phase with burst effect.

In order to assess secondary structure integrity of released insulin, circular dichroism (CD) spectroscopy was carried out. As shown in Fig. 4, The CD spectrum of insulin released from Msp A after day 12 is virtually identical to that of freshly prepared native insulin solution. This means that released insulin preserved its secondary structure. The CD spectrum for the insulin released from Msp B shows weaker

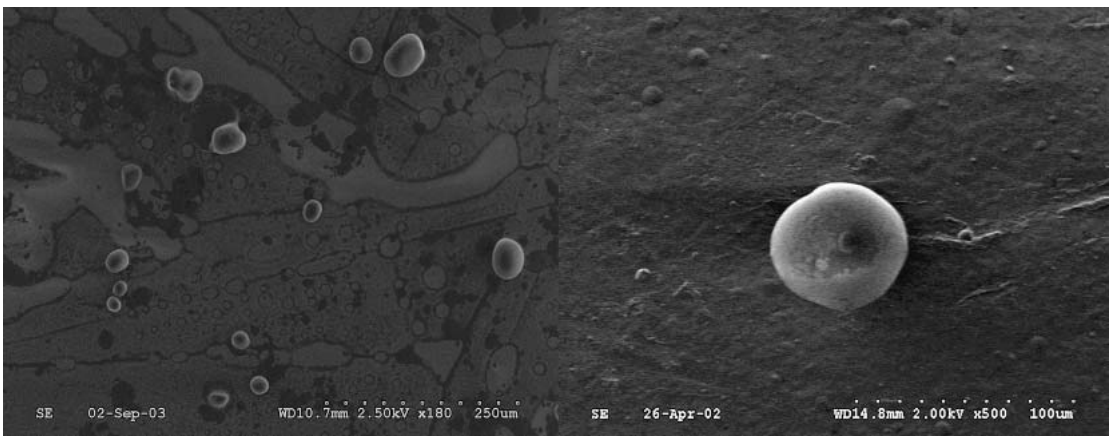


**Fig. 2.** Scanning electron microscopy images of Msp A prepared at the stirring rate of 350 rpm (higher magnification on the right).

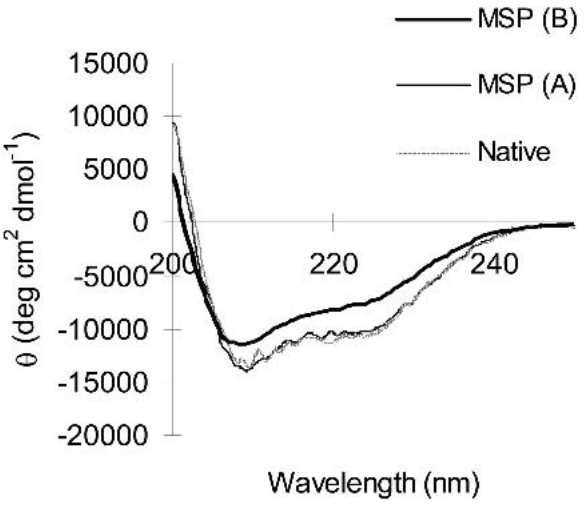


**Fig. 4.** Circular dichroism (CD) spectra of released insulin from Msp A and Msp B with respect to the native insulin solution.

ellipticity values compared to other spectra. This means that insulin in Msp B underwent a processing history under an insulting environment, leading to loss of secondary structure integrity.

The observed release pattern from both types of microspheres lies in the distribution of the protein inside a microsphere, which is associated with the preparation method. In order to see this, FITC-insulin-incorporated microspheres were observed under confocal microscopy. The fluorescence distribution is shown in Fig. 5. For Msp A, homogeneous distribution of fluorescence was observed, whereas Msp B exhibited rather heterogeneous distribution of FITC-insulin. In addition, Msp B shows significant surface fluorescence. Thus, the constant insulin release pattern exhibited by the Msp A may be attributed to this observed homogeneity. It is reported that the constant release of insulin from triblock copolymer hydrogel (16) may be attributed to the hydrophilic/hydrophobic domain structure of the gel. Low-molecular-weight triblock copolymer of PEG and PLGA is known to form micelles at low concentrations (12,14); at higher concentrations, gel forms via packing of the micelles and interaction between hydrophobic phases of the micelles by partial overlap (12). The PLGA-PEG-PLGA used in this study can also form micelles, and gelation is speculated to occur via a similar mechanism (21). Hence, the matrix possesses these microdomains throughout. Thus, a significant

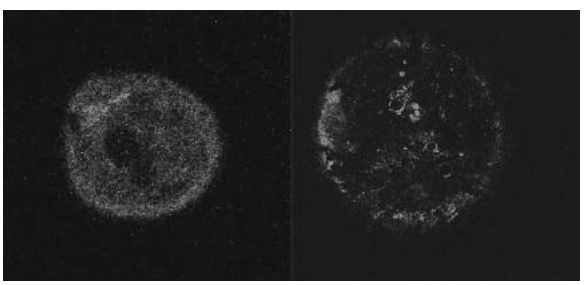

**Fig. 5.** Confocal microscopy images of FITC-insulin-loaded microspheres. (Left) Msp A. (Right) Msp B. Msp A was prepared at the stirring rate of 350 rpm.

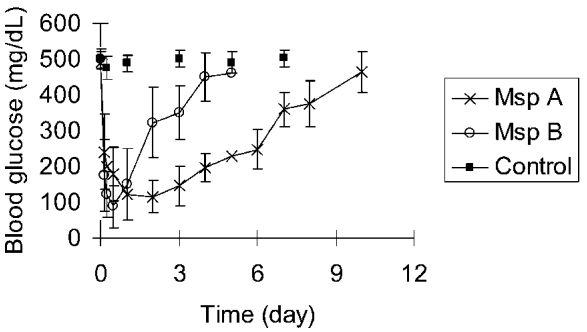


**Fig. 6.** Blood glucose level in STZ-induced diabetic rats after insulin-loaded microsphere injection (260 U/kg, n = 5).

fraction of insulin is incorporated in the hydrophobic domain that allows sustained release of insulin.

On the contrary, based on the confocal image of the Msp B, the initial burst release may reflect the significant fraction of insulin that resides near the surface of the microsphere, and the incomplete release, as mentioned before, is probably because of the aggregated insulin.

Both microspheres were injected s.c. to diabetic rats. As shown in Fig. 6, Msp B exhibited low blood glucose level (~60 mg/dl) in day 1, which rose to control level in 5 days. In some cases, rats died of hyperglycemic shock. In contrast, blood glucose level after injection of Msp A was significantly lower than control level for at least 10 days. Figure 7 shows the plasma insulin level in both cases. Again, the burst effect followed by rapid decrease in insulin level was observed in Msp B, whereas the plasma insulin level in Msp A was kept at a significantly higher level. The duration of *in vivo* insulin release was somewhat shorter compared to that of *in vitro* release, probably because this polymer degrades at higher rate *in vivo* than *in vitro*. Nevertheless, *in vivo* release seems to be consistent with the blood glucose profile shown in Fig. 6 as well as the insulin release pattern observed in the *in vitro* study.

## CONCLUSION

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. Whereas the insulin release

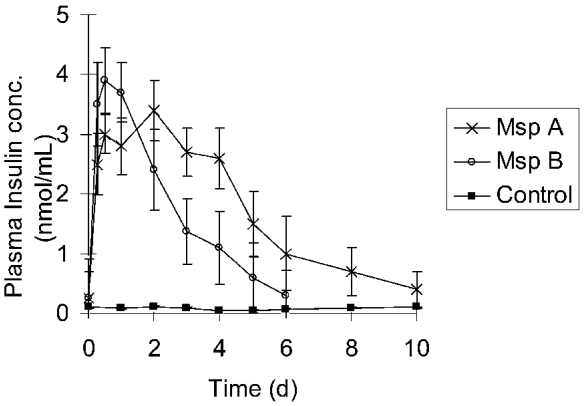


**Fig. 7.** Plasma insulin level in STZ-induced diabetic rats after microsphere injection (n = 5).

from the microspheres prepared using $CH_2Cl_2$ exhibited initial burst release and incomplete release, continuous and nearly complete release (>85%) of insulin *in vitro* was achieved over 3 weeks using aqueous-based PLGA-PEG-PLGA microspheres that contained zinc-crystalline insulin. This *in vitro* release pattern was corroborated by conformation change as shown by CD (Fig. 4) and fluorescence distribution inside the microspheres as shown by confocal images (Fig. 5). *In vivo* studies showed that Msp A exhibited a sustained plasma insulin level over 10 days, whereas Msp B exhibited an initial burst effect and rapid decay after injection of microspheres into STZ-induced diabetic rats. *In vitro* and *in vivo* release profiles of insulin were rather consistent with each other.

## ACKNOWLEDGMENTS

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