CONTROLLED DELIVERY OF INSULIN FROM THERMO-SENSITIVE POLYMER SOLUTION

Biodegradable and temperature sensitive triblock copolymers are capable of releasing a drug over a sustained period of time. The polymers are polyactic acid-polyethylene glycol-polyactic acid (PLA-PEG-PLA) triblock polymers of varying molecular weight and chain lengths which are combined with insulin to provide a patient with zero order insulin release such that a constant delivery of insulin mimics basal insulin released from a healthy pancreas for a period of up to three months. The insulin provided is chemically and conformationally stable and biologically active. Methods for providing sustained-release drugs and a method for treating diabetes are disclosed.

FIG. 6

% Cumulative Release

Time (days)
TITLE: CONTROLLED DELIVERY OF INSULIN FROM THERMOSENSITIVE POLYMER SOLUTION

GRANT REFERENCE
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BACKGROUND OF THE INVENTION
The present invention relates, generally to a controlled delivery system of insulin and a method of providing insulin for extended periods to meet the needs for basal insulin level.

Diabetes mellitus is a term used to describe a collection of metabolic disorders characterized by high blood glucose levels. It results from defects in insulin secretion, action, or a combination of both. Insulin is a polypeptide hormone that decreases the levels of glucose in blood and regulates the metabolism of glucose, fats, and proteins. According to the World Health Organization World Health Report (2006), at least 171 million people suffer from diabetes worldwide and it is estimated that the number will double by the year 2030. The American Diabetes Association national diabetes statistics and reports (2005) show that there has been an increase in diabetes rates in North America over the past 20 years and it affects approximately 20.8 million people, or 7% of the United States population.

The two major types of diabetes are generally referred to as type I and type II. Type I diabetes (Insulin Dependent Diabetes Mellitus, IDDM) is caused by an autoimmune destruction of pancreatic beta cells and leads to absolute insulin deficiency. Type II diabetes (Non-Insulin Dependent Diabetes Mellitus, NIDDM) is not caused by insulin deficiency but rather a reduced sensitivity of cells toward insulin. Complications of diabetes can be life-threatening and rapidly progressive. Examples of macrovascular manifestations include coronary artery diseases (leading to angina or myocardial infarction), stroke, and diabetic myonecrosis. Microvascular complications of diabetes include retinopathy, nephropathy, and neuropathy.

In healthy individuals, insulin is synthesized and stored in the pancreatic beta cells as crystalline hexamers due to the high concentration of zinc and calcium ions. Once the insulin is secreted into the bloodstream, the hexamers are diluted and
dissociate into monomers, the biologically active form. In such healthy individuals, insulin is secreted in both a constant, or basal, manner as well as in response to stimulation. Basal insulin is secreted continuously between meals and throughout the night at a rate of about 0.5 to 1 Unit/hour to maintain serum concentrations of about 5 to 15 μU/ml. Although the basal insulin level is low, it modulates the rate of overnight hepatic glucose and glucose output during prolonged periods between meals. The importance of basal insulin in forestalling or at least postponing the long term complications of diabetes has been well established (Wang, Lancet, 342:129 (1993); Riddle, Am. J. Med., 116, 3S-9S (2004)). In addition to the basal insulin secretion, stimulated insulin secretion occurs in response to a meal, resulting in insulin concentrations of about 60 to 80 μU/ml from just before to 30 minutes after the meal.

Treatment of patients with type I diabetes and many patients with type II diabetes includes insulin replacement therapy. Insulin therapy involves one or more daily doses of intermediate or long-acting insulin injection to satisfy basal insulin requirement, along with insulin injections before meals. Although there are various intravenous and subcutaneous sources of insulin, including insulin pens and pumps, patients are limited to commercially available injectable insulin sources. This type of regimen affects patients’ lifestyle and often leads to poor compliance, pain, mental stress and other concerns and complications associated with therapy requiring ongoing self-injection.

Recent developments have also included oral insulin and inhaled insulin. However, because insulin is a protein, when taken orally it is easily digested by the stomach and gastrointestinal system. Alternatively, inhaled insulin delivered through an inhaler into the lungs was commercially available for a limited period (Exubera®, Pfizer discontinued in 2007). This formulation provided insulin for a period of hours, still requiring patients to continue injecting a long-acting basal insulin. Further disadvantages of the inhaled insulin included manufacturing difficulties resulting in a cost-prohibitive delivery system. As a result, all commercially available insulin formulations must be administered either by subcutaneous or intravenous injection. TABLE 1 shows the major parenteral insulin preparations commercially available (Yadav et al., Indian Pediatr., 43:863-72 (2006)).
TABLE 1.

<table>
<thead>
<tr>
<th>Type</th>
<th>Onset (h)</th>
<th>Duration (h)</th>
<th>Peak (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid acting</td>
<td>0.25</td>
<td>2-4</td>
<td>0.5-1.5</td>
</tr>
<tr>
<td>Short acting</td>
<td>0.5-1</td>
<td>4-6</td>
<td>2-3</td>
</tr>
<tr>
<td>Intermediate acting</td>
<td>2-4</td>
<td>10-16</td>
<td>4-10</td>
</tr>
<tr>
<td>Long acting</td>
<td>6-10</td>
<td>20-24</td>
<td>minimal</td>
</tr>
</tbody>
</table>

The first long acting insulin was developed in 1936 by complexing insulin with protamine and zinc. Ever since, commercially available insulin has contained zinc ions. The addition of zinc salts results in the association of insulin into hexamers which has lower solubility, and therefore longer activity as absorption is impeded (Brange et al., *Diabet. Med.*, 3:532-6 (1986)). Many other proteins undergo similar association upon the addition of zinc, including lactalbumin (Permyakov et al., *J. Protein Chem.*, 10:557-84 (1991)), somatotropin (Stevenson and Hageman, *Pharm. Res.*, 11:S-148 (1994)), and growth hormone (Cunningham et al., *Science*, 253:545-8 (1991)).

The stability of insulin is highly affected by the association state. The hexamer form is the most stable association state, resulting from zinc ions exerting its stabilizing effect by neutralizing negative charges in the center of the insulin hexamer, whereby the hexameric assembly is stabilized. The B13 glutamate charged carboxylate groups in human insulin result in destabilization of the hexamer form and thus neutralizing of those charges by zinc ions promotes insulin hexamer formation. This was confirmed by findings that zinc-free insulin analogue B13-glutamine is hexameric in millimolar concentrations (Hansen, *Biophys. Chem.*, 39:107-10 (1991)). Accordingly, maintaining insulin's stability is an essential feature of a drug delivery system.

The association state of insulin differs based on concentration, in addition to the presence of metal salts, such as zinc. At low concentrations, as in the case of insulin levels in bloodstream, insulin is present in the monomelic form. At higher concentrations, insulin monomers associate into dimers and, if zinc ions are present, into hexamers (Blundell, *Diabetes*, 21:492-505 (1972)). Insulin dimeric structure is formed by non-polar forces and hydrogen bonding. The association of the dimers into hexamers around two zinc ions is associated with the burial of remaining non-polar...
groups (Grant et al., *Biochem. J.*, 126:433-40 (1972)). Each zinc ion coordinates to three BIO histidine residues, promoting the formation of insulin hexamer. It has been shown that calcium could bind to a third site which is composed of six B13 glutamyl carboxylate groups and stabilize the hexamer conformation (Sudmeier et al., *Science*, 212(4494):560-2 (1981); Palmieri et al., *Biochem.*, 27:3387-97 (1988)).


Since the introduction of insulin in 1921 by Banting and Best (Banting and Best, *J. Lab. Clin. Med.*, 7:251-66 (1922)), there has been a continuous search for other means for insulin administration (other than the parenteral route). The major goal has been to achieve a better blood glucose level control with less invasive delivery technologies either through investigating other routes or by reducing the frequency of injections. The simulation of normal basal secretion of insulin is an essential need in the development of a novel insulin delivery system, such that constant amounts of insulin can be continuously released in a patient. The importance of simulating the basal insulin secretion pattern has placed enormous interest in developing controlled delivery systems for insulin which are clinically effective and tolerable by patients.

Polymeric delivery systems have been studied extensively for the controlled release of insulin. Sustained-release formulations of insulin have been reported for the last three decades. It has been shown that insulin loaded into albumin microbead implants was released for up to 3 weeks in diabetic rats (Goosen et al., *Diabetes*, 32:478-81 (1983)). However, this formulation was not injectable and had to be
implanted surgically. Accordingly, the invasive administration methods and other difficulties associated with the polymeric delivery systems of the prior art have failed to provide a desirable mechanism of sustained-release insulin delivery.

Injectable microparticles from poly(D,L-lactide) or poly(D,L-lactide-co-glycolide) have also been investigated for the delivery of insulin (Takenaga et al., *J. Control. Release*, 79:81-91 (2002); Yamaguchi et al., *J. Control. Release*, 81:235-49 (2002); Takenaga at al., *Int. J. Pharm.*, 271:85-94 (2004); Kang and Singh, *Int. J. Pharm.*, 304:83-90 (2005); Liu et al., *J. Mater. Sci. Mater. Med.*, 18:2205-2210 (2007)). However, the use of such microparticles for insulin delivery results in a high burst release followed by a slow release of only one to two week periods. The larger surface areas and size of the microparticles result, at least in part, in the increased burst releases due to the size-dependent nature of the drug delivery. Accordingly, these microparticle delivery systems fail to provide sufficiently controlled release of insulin for delivery of basal insulin levels.

Microspheres composed of alginate have also been developed for insulin delivery. However, such a delivery system fails to provide controlled delivery for any significant period of time as the release was complete over a twenty-four hour period (Silva et al., *Int. J. Pharm.*, 311:1-10 (2006); Reis et al., *Eur. J. Pharm. Sci.*, 30:392-7 (2007)). Microspheres prepared from chitosan showed a biphasic release profile characterized by a high initial burst release (Wang et al., *Int. J. Pharm.*, 311:187-95 (2006); Ubaidulla et al., *J. Pharm. Sci.*, 96:3010-3023 (2007)). In addition to the burst release and short-term delivery of the insulin, microspheres suffer several additional inherent disadvantages. For example, the complicated manufacturing procedure affects insulin stability and there are a variety of hazards and environmental concerns associated with the use of organic solvents like methylene chloride in the manufacturing process and the final drug delivery system. Further, the microspheres have a very low drug loading capacity, and often require surgical implantation, rather than parenteral administration, resulting in an undesirable delivery system. As a result, microspheres have failed to solve the long felt need of suitable insulin delivery compositions.

In addition, various "smart polymers" or biodegradable copolymers with reverse thermal gelation properties have been investigated for the controlled delivery of various biologically active agents, including insulin. However, those of skill in the art have only developed triblock copolymers with controlled release of insulin for a
period of up to two weeks (U.S. Patent No. 6,17,949; Zentner et al., J. Control.
profile exhibited a higher and faster release, most probably due to the high content of
the hydrophilic glycolic acid blocks. U.S. Patent No. 6,17,949 discloses in part the
use of a copolymer delivery system of polylactide-co-glycoside (PLGA) and
polyethylene glycol (PEG) in a triblock formulation (PLGA-PEG-PLGA); however,
such copolymer delivery system is only capable of achieving a continuous release of
insulin for a period of up to approximately one week. In addition to the short release
period achieved, the structural and chemical stability of the insulin delivered was not
known. As a result, a stable delivery system for basal insulin levels over controlled
periods of two to three months has not been achieved by those skilled in the art,
including U.S. Patent No. 6,17,949. Accordingly, there remains a significant need in
the art for controlled release of conformationally and chemically stable insulin for
periods of up to two to the three months for the treatment of diabetes.

Accordingly, the various embodiments of the present invention provide for a
thermo-sensitive triblock copolymer of polylactide (PLA) and PEG (PLA-PEG-PLA)
providing improved controlled release of insulin, method of making the compositions
and methods of use for the same, solving a long felt need for controlled release
therapies for insulin.

It is thus an object of the present invention is to develop biocompatible and
biodegradable delivery systems which can be used for the delivery of basal insulin for
treating diabetes.

Yet another object of the present invention is to develop controlled release
injectable formulations which can deliver insulin in a conformationally stable and
biologically active form to meet the basal requirements of insulin.

A still further object of the present invention is to develop injectable thermo-
sensitive controlled delivery systems for basal insulin delivery using different
polylactic acid polyethylene glycol-polylactic acid (PLA-PEP-PLA) triblock
copolymers.

A still further object of the present invention is to develop injectable delivery
systems for basal insulin with adjustable release profiles based on the polymer
composition and concentration.
The method and means of accomplishing each of the above objectives, features and/or advantages as well as others will become apparent from the detailed description of the invention and claims which follows hereafter.

5 SUMMARY OF THE INVENTION

In one embodiment of the present invention, a polymeric system for the controlled release of insulin is developed. The basal level of insulin secretion of healthy animals, for examples humans not suffering from diabetes, is mimicked through the controlled delivery system of the present invention which overcomes the problems in the art of suboptimal release patterns, characterized by a high initial burst followed by a diminishing release rate over a period of few days.

According to another embodiment of the present invention, a zero order release of insulin from developed polymeric systems over up to three months is achieved. Temperature sensitive delivery systems composed of poly(lactide)-polyethylene glycol-poly(lactide) (PLA-PEG-PLA) triblock copolymers are synthesized and used as drug delivery systems and exhibit sol-gel transition in response to temperature changes. At room temperature, the delivery systems exist in solution form and once injected into the body, it solidifies to form a depot at site of injection. Further, in vitro and in vivo assays show the systems are biodegradable which omits the need for removal once the drug is released (unlike implants). Biocompatibility assays demonstrated that the fabricated delivery systems are biocompatible.

According to yet another embodiment of the present invention, the developed polymeric delivery systems is capable for use for the controlled delivery of insulin complexed with zinc acetate, to favor the formation of the less soluble and more stable insulin hexamer form, for periods of up to three months with concurrent reduction in blood glucose levels.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagram illustrating the chemical synthesis of PLA-PEG-PLA triblock copolymers.

FIG. 2 is the $^1$H-NMR spectrum for triblock copolymer 1 (PLA-PEG-PLA).

FIG. 3 is the gel permeation chromatography (GPC) spectrum of triblock copolymer 1 (PLA-PEG-PLA).
FIG. 4 is a phase diagram of PLA-PEG-PLA triblock copolymers. Key: (A) copolymer 1 (1496-1500-1496); (α) copolymer 2 (1584-1500-1584).

FIG. 5 illustrates in vitro release of lysozyme from delivery systems containing triblock copolymer 1 (1496-1500-1496) (n = 4). Key: Copolymer concentrations of (■) 25%, (●) 30%, and (▲) 40%.

FIG. 6 illustrates in vitro release of lysozyme from delivery systems containing triblock copolymer 2 (1584-1500-1584) (n = 4).

FIG. 7 illustrates the effect of zinc addition on in vitro release of insulin from 40% (w/w) copolymer 1 (1496-1500-1496) (n = 4). Key: (■) zinc acetate and (A) no zinc salt added.

FIG. 8 illustrates the effect of polymer concentration on in vitro release of insulin from triblock copolymer 1 (1496-1500-1496) (n = 4). Key: copolymer concentration of (■) 30% and (▲) 40%.

FIG. 9 illustrates the effect of polymer concentration on in vitro release of insulin from triblock copolymer 2 (1584-1500-1584) (n = 4). Key: copolymer concentration of (■) 30% and (A) 40%.

FIG. 10 illustrates the effect of insulin loading on the in vitro release of insulin from triblock copolymer 2 (1584-1500-1584) (n = 4). Key: Insulin amount incorporated (A) 5 mg and (■) 25 mg.

FIG. 11 illustrates DSC thermograms of released insulin after one month from copolymer 2 thermo-sensitive system (2.5% w/v insulin): fresh insulin (solid line), and released insulin (dashed line).

FIG. 12 illustrates CD spectra of released insulin from copolymer 2 thermo-sensitive system (2.5% w/v insulin): fresh insulin (solid line), one month released insulin (dashed line), and two months released insulin (dotted line).

FIG. 13 illustrates MALDI-TOF mass spectroscopy of fresh insulin (control).

FIG. 14 illustrates MALDI-TOF mass spectroscopy of released insulin (1 month) from thermo-sensitive delivery system (40% copolymer 2).

FIGS. 15A and 15B illustrate MALDI-TOF spectroscopy of released insulin after (A) one month and (B) two months from thermo-sensitive delivery system (40% copolymer 2).

FIGS. 16A and 16B illustrate serum insulin concentration (A) and blood glucose level (B) following subcutaneous administration of insulin solution (n=6).
FIGS. 17A and 17B illustrate serum insulin concentration (A) and blood glucose level (B) following subcutaneous administration of thermo-sensitive polymeric delivery systems in SD rats (n=6). Key: (■) blank thermo-sensitive delivery systems (control), (●) thermo-sensitive delivery systems composed of 40% (w/v) copolymer 1 (1496-1500-1496), and (A) thermo-sensitive delivery systems composed of 40% (w/v) copolymer 2 (1584-1500-1584).

FIGS. 18A and 18B illustrate in vitro biocompatibility of thermo-sensitive copolymer 1 (1496-1500-1496) extracts (A) prepared at 37°C and (B) prepared at 70°C (n=8). [PBS = Phosphate buffered saline, PE = Polymer extract]

FIGS. 19A and 19B illustrate in vitro biocompatibility of thermo-sensitive copolymer 2 (1584-1500-1584) extracts (A) prepared at 37°C and (B) prepared at 70°C (n=8). [PBS = Phosphate buffered saline, PE = Polymer extract]

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Unless mentioned otherwise, the techniques employed or contemplated herein are standard methodologies well known to one of ordinary skill in the art. The materials, processes and examples described in the description of the invention are illustrative only and not intended to be limiting to the scope of the invention in any manner. Many modifications and other embodiments of the inventions set forth herein will come to mind to one skilled in the art to which these inventions pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the inventions are not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims.

As used herein, the following terms shall have the following meanings:

"Biocompatible" means the triblock copolymer of the present invention does not result in any significant reaction upon injection into a human body.

"Biodegradable" means the process by which organic substances are broken down in the body and degrade once injected into a human body, such as the triblock copolymer degrading or being broken down in vivo to form small non-toxic
components, such as carbon dioxide and water, allowing the injection of the delivery system without requiring any subsequent surgical removal.

"Biologically active agent" means any pharmacologically and/or pharmaceutically agent, including recombinant or naturally-occurring proteins from either human or animal source, useful for prophylactic and/or therapeutic purposes. Such agents can be natural, synthetic or derivatives thereof either source. A person of ordinary skill in the art will be readily able to utilize any biologically active agent with the compositions contained herein the detailed description of the invention.

"Depot" means a drug delivery system, typically injected as a subcutaneous or intramuscular injection, of a pharmacological agent capable of consistently releasing an active compound over an extended period of time.

"Gel-to-sol transition" means the transition of the insulin of the present invention in its gel state back to its lower viscosity solution state due to the exposure to cooled temperatures. The transition back to an aqueous solution occurs while maintaining chemical and structural conformation of the insulin.

"Therapeutically effective amount" means that amount of the composition or biologically active agent which provides a therapeutic benefit in the treatment, prevention, or management of one or more therapeutic conditions, namely diabetes. The phrase further refers to the amount of insulin in the delivery system of the present invention that provides a therapeutic benefit in the management or treatment of diabetes, resulting in observable and/or measureable improvement over the baseline clinically observable signs and symptoms of one or more forms of diabetes. One of ordinary skill in the art is familiar with such signs and symptoms and measurements of improvement. It is further understood by those of ordinary skill in the art that this amount varies depending on the drug used, such as the type of insulin used, type of diabetes being treated, and characteristics of the patient.

"Sol-to-gel transition" means the transition of the insulin solution to a semi-solid gel state due to an increase in viscosity when exposed to a temperature change. The insulin is an aqueous solution at low temperatures (refrigeration) and upon injection to a human body (warm-blooded having a temperature of 37°C) becomes a gel through the process of gelation. The transition can further proceed to forming a precipitate in the event it is exposed to temperature significantly exceeding 37°C. However, such temperatures are not compatible with life in a human being.
Accordingly, the insulin of the present invention remains in a gel state for a period of up to three months.

The present invention provides for an injectable thermo-sensitive controlled polymeric delivery system for basal insulin delivery using different polylactic acid-polyethylene glycol-polylactic acid (PLA-PEG-PLA) triblock polymers. The biodegradable delivery system provides for the controlled release of insulin to mimic basal levels secreted by healthy individuals not suffering from diabetes, such that a zero order release of insulin from over two and three months is achieved. The polymeric drug delivery system exhibits sol-to-gel transition in response to temperature changes, wherein at room temperature the delivery systems exists in solution form and once injected into the body the delivery system solidifies to form a depot at site of injection.

In one embodiment of the present invention, a pharmaceutically acceptable injectable and/or implantable delivery system is described. In yet another embodiment of the present invention, a pharmaceutically acceptable carrier for insulin or other biologically active agents is described. The carrier may be prepared by forming a mixture of the biologically active agent(s) and the acceptable carriers discussed hereunder. A suitable pharmaceutically acceptable carrier may include combinations of temperature sensitive polymers as will be readily apparent to one of ordinary skill in the art based upon the detailed description of the invention.

The precise temperature sensitive polymers used in the delivery system may include varying combinations of polylactic acid (PLA), wherein PLA is alternatively referred to as a poly(α-hyrdroxoy acid), poly(lactide), poly(d,l-lactide), or poly(l-lactide), and polyethylene glycol (PEG), wherein PEG is alternatively referred to as polyethylene oxide (PEO) or poly(oxyethylene), which terms are to be used interchangeably for purposes of the present invention. The polymers are ideal for the controlled delivery of biologically active agents, such as insulin, due to the formation of both water soluble (PEG) and water insoluble (PLA) components of the delivery system capable of detracting water and forming loops, such that micelles are formed for a delivery system for the biologically active agent. Micelle formation is most preferably obtained using a delivery system comprising polylactic acid-polyethylene glycol-polylactic acid (PLA-PEG-PLA) triblock polymers.

Preferred embodiments of the PLA-PEG-PLA triblock polymers include conformations having molecular weight distributions with average molecular weights
for the PLA polymers ranging from 800 to 2300, and average molecular weights for the PEG polymers ranging from 400 to 1500, more preferably from 1400-1500-1400 (PLA-PEG-PLA) to 1600-1500-1600 (PLA-PEG-PLA), and most preferably from either 1496-1500-1496 (PLA-PEG-PLA), as will be referred to herein as Copolymer 1, or 1584-1500-1584 (PLA-PEG-PLA), as will be referred to herein in as Copolymer 2. Further, the triblock polymers preferably have an average molecular weight determined by GPC ranging from 3100 to 7200, more preferably from 6900 to 5000, and most preferably from 6935 to 5009 as set forth in Copolymers 1 and 2, respectively. The increase in polymer chain length had markable impacts on the solubility of the copolymer delivery system. For example, as the PLA chain length increase the increased hydrophobicity precludes the coblock polymers from being water soluble. Accordingly, the preferred embodiments arrive at a precise length where the coblock polymers can form an aqueous solution with the biologically active agent, without the required use of any organic solvents, as previously required by those skilled in the art. According to the present invention by increasing the length of the coblock polymers, an implantable delivery system is created.

The triblock polymers of the present invention are thermo-sensitive such that variations in temperature result in sol-to-gel and gel-to-sol transitions of the polymers containing the biologically active agent. Preferably the triblock polymers demonstrate concentration-dependent gelation in response to temperature changes, such that as polymer concentration increases, sol-to-gel transition temperature decreases and gel-to-sol transition temperature increases. The polymer transition between solution state, gel state, and precipitate, such that below the lower critical transition temperature (LCST) (i.e., refrigeration temperatures), the hydrogel exists in the solution state, whereas above the LCST it exists in the gel form (i.e., upon injection to a human body) and, once temperature rises above upper critical transition temperature (UCST) (i.e., upon heating or boiling the solution), the polymer precipitates and hydrogel exists in solution state again.

The gelation of thermo-sensitive delivery systems is driven by entropy where the increase in temperature decreases the hydration of the PEG blocks, resulting in lower water-polymer hydrogen bonding and, consequently, the formation of a more hydrophobic character (Jeong et al., Colloid Surfaces B, 16:185-93 (1999)). The delivery systems of the present invention demonstrating the release of proteins from thermo-sensitive triblock copolymers is highly dependent on copolymer structure.
The ratio of hydrophilic (PEG) and hydrophobic (PLA) segments, hydrophobicity, and block length are crucial factors. Small changes in these factors can lead to drastic effects on sol-to-gel formation and drug release.

For example, lactic acid, a more hydrophobic component than glycolic acid (due to the methyl group presence in lactic acid), results in lactide-rich PLGA copolymers being more hydrophobic, absorbing less water, and subsequently degrading more slowly (Mil and Nixon, J. Microencapsul., 7:297-325 (1990); Cohen et al., Int. J. Technol. Assess. Health Care, 10:121-30 (1994)). Similar results have been reported when PLGA-PEG-PLGA thermosensitive delivery systems with varying LA/GA ratios were used for the delivery of 5-fluorouracil (Qiao et al., Int. J. Pharm., 294:103-12 (2005)). Furthermore, increasing LA/GA ratio decreases burst release and extends the release period of BSA from PLGA microspheres (Sah and Chien, Drug Dev. Ind. Pharm., 19:1243-63 (1993); Sah et al., J. Control. Release, 30:201-11 (1994)).

The precise biologically active agents used in the delivery system of the present invention may vary. Various biologically active agent(s) may be used, including various peptides, proteins and hormones. Further, those skilled in the art of formulation know the precise percentage of the active will vary depending upon a number of factors, including the type of biologically active agent used, what form the agent is used in (i.e., whether the agent is used in free base or salt form), the type of condition being treated and the severity of its symptoms, etc.

In one embodiment, insulin is selected as the biologically active agent. Various forms of insulin may be used in varying embodiments of the present invention. For example, insulin shall be understood by those of skill in the art to include all forms of insulin, including without limitation, insulin, insulin analogs, and recombinant insulin, synthetic, human or animal source insulin, as well as mixtures of types of insulin. The precise quantity of insulin is then mixed with zinc acetate in a preferred ratio of insulin to zinc ion of about 1:5. This preferred embodiment of zinc acetate is added to insulin delivery systems at a molar ratio of 1:5 insulin hexamer to zinc ion in order to favor the formation of the more stable and less soluble hexamer form. The delivery of insulin in the hexameric form allows for longer storage periods and improves the controlled delivery of the composition due to the chemical stability of the hexameric form. The insulin is injected in its hexameric forms and
thereafter dissociates into the monomer form in order to provide its physiological and pharmaceutical effects on blood glucose levels.

The actual insulin percentage selected will vary according to factors known to those of ordinary skill in the art. However, the percentage of insulin will generally be within the range of from about 0.5% to 10% by weight of the delivery system of the present invention, with about 4.5% to 9% by weight being preferred, and approximately 6% more preferred. Varying amounts of insulin can be loaded for different doses. The dosages of insulin supplied will also vary greatly depending upon a variety of factors, including for example, type of diabetes being treated, patient response to insulin, etc., but will generally range from about 0.5 - 1 units/hour, delivering an average of 12 - 24 units/day of basal insulin, hi one embodiment of the present delivery system, the injectable insulin solution delivers 27 units of insulin per 1 mg solution. Accordingly, a three month controlled delivery system of such embodiment requires from approximately 45 mg to 90 mg of insulin to be released in zero order to provide a patient with a continuous 0.5 to 1 units of insulin per hour constantly for a period of up to three months.

The delivery system containing the biologically active agent may also contain other active agents that are compatible with the insulin or other biologically active agent, and the zinc acetate and polymers of the co-block delivery system, if desired. Moreover, in addition to additional active agents, the delivery system may further contain other inactive agents, including suitable excipients and auxiliaries which facilitate processing of the biologically active agents into preparations and delivery purposes as may be apparent to those of ordinary skill in the art. Such additional inactive agents may include, without limitation, pharmaceutically acceptable diluents, excipients, wetting agents, buffering agents, suspending agents, lubricating agents, adjuvants, vehicles, delivery systems, emulsifiers, disintegrants, absorbents, preservatives, surfactants, and/or colorants that would be suitable for use in a pharmaceutical composition. For example, aqueous injection suspensions may contain substances which increase the viscosity of the suspension, including for example, sodium carboxymethyl cellulose, sorbitol and/or dextran, optionally the suspension may also contain stabilizers. Such examples are given for illustrative purposes only and are in no way intended to limit the invention.

Included in the various embodiments of the present invention are various conditions which occur during preparation and release of insulin from delivery
systems affecting its stability. The present invention improves upon methods
previously used in the art, such that the presence of organic solvents, pH changes in
the microenvironment vicinities in the depot, the presence of salts in the release
medium, and manufacturing methods resulting in increased temperature changes, such
as boiling insulin, do not occur in any embodiments of the present invention. (Banting
and Best, J. Lab. Clin. Med., 7:251-66 (1922)), agitation causing aggregation and
adsorption (Sluzky et al., Proc. Natl. Acad. Sci., 88:9377-81 (1991); Sadhale and
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adsorption (Sluzky et al., Proc. Natl. Acad. Sci., 88:9377-81 (1991); Sadhale and
Shah, Int. J. Pharm., 191:51-64 (1999)). The elimination of such manufacturing
methods are critical, as studies have shown, for example, a significant loss of
secondary structure of insulin that is proportional to increasing temperatures. (Ettinger
and Timasheff, Biochemistry, 10:824-31 (1971); Arora et al., Protein Sci., 13:2429-36
(2004); Huus et al., Biochemistry, 44:1 1171-7 (2005); Dzwolak et al., Biochemistry,
44:8948-58 (2005)). In one embodiment of the present invention, DSC thermograms
showed the released insulin had lower ΔH and Tm compared to control (i.e., fresh
insulin). CD results showed a reduction in the magnitude of both minima at 209 nm
and 222 nm in released insulin compared to control.

The partial reduction in conformational stability and secondary structure is
common in released peptide and protein samples (Duncan et al., J. Control. Release,
110:34-48 (2005)). The reduction in released insulin conformational stability could
be attributed to insulin prolonged existence in agitated media at 37°C temperature,
which is not ideal for insulin stability (see e.g., Oliva et al., Int. J. Pharm., 143:163-70
(1996)). However, the conservation of most of denaturation peak in DSC thermogram
and CD characteristic spectrum demonstrate the secondary structure and
conformational stability were greatly preserved during the present invention's delivery
system preparation and throughout release studies. In a further embodiment of the
present invention, the injectable insulin solution is stable in aqueous form for periods
of approximately three to four years if maintained under proper storage (i.e.,
refrigeration) without any degradation of the insulin protein.

The correlation between protein unfolding and chemical stability is not
straightforward as many factors affect protein chemical stability (Wang, Int. J.
Pharm., 185:129-88 (1999)). Yet, chemical degradation, which takes place in protein
domains that are protected in native state, occurs at higher rates when the protein is
denatured (Kossiakoff, Science, 240:191-4 (1988); Xie and Schowen, J. Pharm. Sci.,
88:8-13 (1999)). It has been shown that there is a high correlation between DSC
results and insulin B3 deamidation products (Huus et al., Biochemistry, 45:4014-24 (2006)). Yet, DSC data failed to predict the rate of covalent insulin dimer formation. In addition, since DSC measures thermal stability, it cannot be used to predict chemical stability at low temperatures.

Insulin undergoes many chemical modification reactions depending on the surrounding environment. However, the effect on biological activity varies depending on the nature of the forming products. Deamidation products have almost the same biological activity as native insulin, while covalent insulin dimers (CID) and hydrolysis products (A8-A9) exhibit only 15% and 2% relative potency, respectively (Brange et al., Pharm. Res., 9:715-26 (1992)). In one embodiment of the present invention, MALDI results showed that insulin released from temperature-sensitive delivery systems did not show peaks for acylation, CID, or hydrolysis products and the spectra were comparable to fresh insulin spectrum. A small signal denoting the formation of cyclic imide by the loss of a water molecule was observed. This reaction usually follows deamidation reactions and may lead to the formation of IsoAsp-insulin (Houchin et al., J. Control. Release, 112:1 11-9 (2006)). However, two months released insulin exhibited an increase in the cyclic imide signal which denotes an increase in deamidation reactions. Additional hydrolysis degradation products signals (5217.1 and 5070.2 Da), which correspond to B24-B25 and B25-B26 split products, respectively, were observed and likely a result of the prolonged presence in the shaking release media at 37°C. However, in vivo studies show that released insulin is rapidly absorbed.

In one embodiment of the present invention, the insulin release from the thermo-sensitive copolymer delivery systems has lower initial burst release with zero order release, dependent upon the concentration of copolymers and the polymer chain length. In a preferred embodiment, the burst release of insulin decreases as concentration of the copolymer delivery system increases. Copolymer concentrations may vary from approximately 25% to 40%, more preferably from 30% to 40% resulting in high zero order correlation causing constant drug release from controlled delivery systems. The precise polymer concentration is essential to the present invention, requiring a sufficiently high concentration in order for the sol-to-gel transition to occur when the insulin solution is injected in to patient. Similarly, there is an apparent upper limit to the polymer concentration, such that the insulin solution remains injectable, rather than requiring surgical implantation. In another preferred
embodiment, improved insulin release profiles are obtained using a delivery systems containing 40% (w/w) copolymer 2 (1584-1500-1584), with an increased chain length, rather than copolymer 1 (1496-1 500-1496). The increased hydrophobic character of copolymer 2 causes decreased water diffusivity, slower degradation, and lower release rates.

In another embodiment of the present invention, the addition of metal ions further lowers burst release of insulin from the delivery system. The addition of metal ions reduces insulin association in the PEG domain as a result of lowered insulin solubility, causing less insulin available for an initial burst release. The majority of incorporated insulin is associated with the PLA polymer and then released during the degradation phase, rather than an initial burst release. Various metal ions could be utilized for complexation with insulin, including for example calcium, magnesium or zinc. A more preferable embodiment includes the addition of zinc ions to control the release of insulin for extended periods due to increased bioavailability of insulin compared to subcutaneous injections of insulin solutions. In a preferred embodiment of the invention, temperature-sensitive based delivery systems increase insulin bioavailability by 7.59 to 10.91 fold. Further, the delivery systems allow longer term release of the insulin. In a preferred embodiment the thermo-sensitive delivery systems control the release of insulin over one to three month periods, more preferably the insulin release is controlled for two to three month periods.

EXAMPLES

Embodiments of the present invention are further defined in the following non-limiting Examples. It should be understood that these Examples, while indicating certain embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the embodiments of the invention to adapt it to various usages and conditions. Thus, various modifications of the embodiments of the invention, in addition to those shown and described herein, will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.
EXAMPLE 1

The synthesis of the thermo-sensitive PLA-PEG-PLA triblock copolymer followed the methods of Zentner et al. (J. Control. Release, 72:203-15 (2001)) with modifications according to the significant differences in polymer formation, chain length and molecular weight necessary in order to provided controlled release of insulin over a period of up to three months. FIG. 1 shows the chemical synthesis of the triblock copolymer by ring-opening method (Chen et al., Int. J. Pharm., 288:207-18 (2005)). Under dry nitrogen, PEG was dried at 100°C in a three-necked flask for 30 minutes. D,L-lactide was added and the reaction mixture was heated for 30 minutes. After melting of the reactants, 0.03% w/w of stannous 2-ethylhexanoate was added, and the reaction mixture was further heated at 120°C for 12 hours. Crude polymer was dissolved in ice-cold water (from about 5-8°C) and then reheated for purification. Finally, freeze-drying was performed to remove residual water.

EXAMPLE 2

This example describes the characterization of the triblock copolymer delivery system performed utilizing 1H-NMR analysis and gel permeation chromatography (GPC). 1H-NMR was first used to determine the structure of triblock copolymers (FIG. 2). Spectra were recorded on a Varian spectrometer at 300 MHz and 25°C. Deuterated chloroform (CDCl₃) was used as a solvent and tetramethylsilane (TMS) signal was taken as the zero chemical shift. Number average molecular weight was determined by integrating the signals pertaining to different chemical groups such as the peaks from CH and CH₃ of LA, and CH₂ of ethylene. Chemical shift (δ) signals originating from different protons were used to confirm the structure. The signals pertaining to PLA-PEG-PLA were found at δ = 5.20 ppm (CH of LA), 1.55 ppm (CH3 of LA), 3.65 ppm (CH2 of ethylene glycol), and 2.35 ppm (OH of LA). The spectrum was similar to spectra reported in literature for triblock copolymers (Jeong et al., Colloid Surfaces B, 16:185-93 (1999)).

GPC was further used to determine the structure of triblock copolymers, including the molecular weight and molecular weight distribution (Waters 515, Milford, MA). The apparatus was equipped with a Waters 2410 refractive index detector and two Styrage® HR4E and HR5E columns (Milford, MA). The samples were run at 20°C using tetrahydrofuran (THF) as an eluent at a flow rate of 1 ml/min. GPC instrument was calibrated with Polystyrene standards. FIG. 3 represents GPC
retention spectrum and analysis for copolymer 1. The retention time of the triblock copolymer was around 17 minutes while the other peaks in the chromatogram were from the solvent. All GPC spectra showed a symmetric peak and had a relatively narrow molecular weight distribution. The polydispersity index values of all copolymers were found to be close to 1, confirming the synthesized polymers have uniform distribution of molecular weight with high purity. The characteristics of the triblock copolymers from 1H-NMR and GPC results are listed in TABLE 2.

<table>
<thead>
<tr>
<th>Triblock copolymer</th>
<th>NMR</th>
<th>GPC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mn(^{a})</td>
<td>Mn(^{b})</td>
</tr>
<tr>
<td>Copolymer 1</td>
<td>1496-1500-1496</td>
<td>6227</td>
</tr>
<tr>
<td>Copolymer 2</td>
<td>1584-1500-1584</td>
<td>4709</td>
</tr>
</tbody>
</table>

\(^{a}\) Number average molecular weight determined by NMR.  
\(^{b}\) Number average molecular weight determined by GPC.  
\(^{c}\) Weight average molecular weight determined by GPC.  
\(^{d}\) Polydispersity index determined by GPC.

EXAMPLE 3

This example describes phase diagram experiments carried out to determine the sol-to-gel transition temperatures. The triblock copolymers were dissolved in distilled water at different concentrations (10, 15, 20, 25, 30, 35, 40% w/w). After equilibration at 4°C overnight, vials containing copolymer solutions were immersed in a water bath equilibrated at 10°C and temperature was increased to 60°C at 2°C /step increments (Jeong et al., Colloid Surfaces B, 16:185-93 (1999)). The sol-to-gel transition was determined by inverting the vial horizontally after keeping the sample at a constant temperature for 10 minutes.

Phase transition diagrams of the triblock copolymer aqueous solutions are depicted in FIG. 4. Polymers showed concentration-dependent gelation in response to temperature changes. As polymer concentration increases, sol-to-gel transition temperature decreases and gel-to-sol transition temperature increases. As temperatures increased from 10 to 65°C, the polymer solution presented three physical states; solution state, gel state, and precipitate. Below the lower critical transition temperature (LCST), the hydrogel exists in the solution state. Above
LCST, it exists in the gel form and, once temperature rises above upper critical transition temperature (UCST), the polymer precipitates and hydrogel exists in solution state again.

EXAMPLE 4

The formulation of the copolymer delivery systems, incorporating the biologically active agent was prepared by first dissolving the prepared copolymers into water. Protein was added to the copolymer solution and homogenized at 8000 rpm for 30 seconds at room temperature to form a clear solution or dispersion. Then the biologically active agent is formulated prior to combining it with the copolymers. For the insulin formulation, the known quantity of insulin was mixed with zinc acetate in the ratio of insulin to zinc ion (1:5). The insulin-zinc ion mixture was then homogenized with aqueous copolymer solution at 8000 rpm for 30 seconds. The composition of the insulin delivery systems is listed in TABLE 3.

<table>
<thead>
<tr>
<th>Co-polymer</th>
<th>Copolymer concentration (% w/w)</th>
<th>Insulin hexamer: Metal ion</th>
<th>Insulin (%w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>1:5</td>
<td>0.5</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>1:5</td>
<td>0.5</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>—</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>1:5</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>1:5</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>1:5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

EXAMPLE 5

This example describes the methods of the in vitro release studies performed on the copolymer delivery systems with insulin. The in vitro release studies utilized lysozyme to study the kinetics from the thermo-sensitive copolymer delivery systems.

First, protein was added to different copolymer aqueous solutions and homogenized at 8000 rpm for 30 seconds to form clear solutions at room temperature. A 1 ml polymer solution (containing the dissolved drug) was injected into a 20 ml test tube and the test tubes were kept in a water bath at 37°C. After formation of the gel,
15 ml of pre-warmed phosphate buffered saline (pH 7.4) was added to the test tube as release medium. Unless otherwise stated, zinc acetate was added to insulin delivery systems at a molar ratio of 1:5 insulin hexamer to zinc ion to favor the formation of the more stable and less soluble hexamer form. All test tubes were continuously kept in a shaker water bath at 37°C and 35 rpm. Five milliliter samples were removed from the media at different time points. The removed volume was replaced with fresh PBS. The samples were centrifuged at 4229 x g for 30 minutes and then diluted with PBS.

MicroBCA protein assay was utilized to determine the amount of protein released in the samples (Smith et al., Anal. Biochem., 150:76-85 (1985)). Briefly, one hundred and fifty microliters of the diluted sample were placed in each well of the microplate. One hundred and fifty microliters of working reagent solution prepared by mixing MicroBCA reagents A, B, and C in the ratios of 50, 48, and 2 were added to the wells. The microplate was covered, placed on a shaker for 30 seconds, and incubated at 37°C for 2 hours. The plate was then cooled to room temperature and absorbance was measured at 562 nm by MRX-Microplate Reader utilizing Revelation® software. Samples from formulations containing thermo-sensitive delivery systems without protein were used as a blank control for absorbance. The amount of protein released in the samples was obtained from the standard curve and corrected for sample removal (Hayton and Chen, J. Pharm. ScL, 71:820-1 (1982)).

Studies with thermo-sensitive triblock copolymers included the use of a model protein, lysozyme. Low burst release was observed from delivery systems composed of copolymer 1 (FIG. 5), notably 20.93±2.61, 7.28±2.65, and 5.25±0.80 burst release of lysozyme from delivery systems containing 25%, 30%, and 40% copolymer concentrations. Delivery systems composed of 25% copolymer concentration showed best fit for Higuchi model while systems made of 30% and 40% copolymer concentration exhibited best fit for zero order model with \( r^2 \) values of 0.98 and 0.99, respectively (TABLE 4). The high zero order correlation is highly desired to ensure constant drug release from controlled delivery systems.

**TABLE 4.**

<table>
<thead>
<tr>
<th>Polymer concentration (% w/w)</th>
<th>Zero order ( r^2 )</th>
<th>First order ( r^2 )</th>
<th>Higuchi ( r^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Further, FIG. 6 shows *in vitro* release profile of lysozyme from delivery systems containing 40% (w/w) copolymer 2 (1584-1500-1584). The increase in PLA chain in copolymer 2 (compared to copolymer 1, 1496-1500-1496) increased the hydrophobic character which led to lower water diffusivity, slower degradation, and, consequently, lower release rate. The release of lysozyme was controlled over a period of one month. The two copolymers were then used for the formulation of insulin delivery systems. TABLE 5 lists the composition of insulin delivery systems made of copolymers 1 and 2 and insulin *in vitro* release kinetics.

### TABLE 5.

<table>
<thead>
<tr>
<th>Co-hexamer</th>
<th>Copolymer concentration (% w/w)</th>
<th>Insulin: Insulin zero order</th>
<th>First order</th>
<th>Higuchi</th>
<th>r^2</th>
<th>r^2</th>
<th>r^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metal ion</td>
<td></td>
<td>Zero order r^2</td>
<td>First order r^2</td>
<td>Higuchi r^2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>0.5</td>
<td>0.97</td>
<td>0.82</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>0.5</td>
<td>0.99</td>
<td>0.86</td>
<td>0.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>0.5</td>
<td>0.95</td>
<td>0.79</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>0.5</td>
<td>0.97</td>
<td>0.79</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>0.5</td>
<td>0.99</td>
<td>0.82</td>
<td>0.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>2.5</td>
<td>0.95</td>
<td>0.77</td>
<td>0.97</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**EXAMPLE 6**

This example describes the effects of metal ion addition and polymer concentration on insulin release from copolymer 1-based thermo-sensitive delivery systems are shown in FIG. 7 and FIG. 8, respectively. FIG. 7 demonstrates that the addition of zinc lowered the burst release with zero order r^2 value of about 0.99 compared to a value of about 0.95 when no zinc was added and resulted in a
controlled release of insulin for two months. The addition of zinc ions shifted best fit for release kinetics from Higuchi to zero order model, which is highly desired to assure release of insulin at a constant rate regardless of insulin concentration in the depot.

Increasing the polymer concentration from 30% to 40% reduced the burst release and improved release kinetics (FIG. 8 and TABLE 5). Similar results were observed when copolymer 2 (1584-1500-1584) was used (FIG. 9 and TABLE 5). Increasing the polymer chain length to 1584-1500-1584 (compared to 1496-1500-1496) further extended the release period. The insulin was released from delivery systems containing 40% copolymers 1 and 2, to which zinc ions were added at the molar ratio 1:5 insulin hexamer to zinc ion, showed a zero order ($r^2=0.99$) release of insulin over periods of two and three months, respectively. Test results demonstrate that increasing polymer concentration and metal ion addition improve the release profile by lowering, and even eliminating, the burst release usually associated with thermo-sensitive delivery systems. It appears the addition of metal ions reduced insulin association in the PEG domain by lowering insulin solubility, resulting in less insulin available for the initial burst release. Rather, most of the incorporated insulin is associated with PLA and released during the degradation phase, resulting in the controlled release of the insulin to stimulate the basal insulin levels.

An important aspect in the use of controlled release systems as proteins delivery devices is the ability of loading different doses of proteins. This is mainly to count for intra- and inter-individual drug requirement variations. By increasing the insulin loading dose in delivery systems containing copolymer 2 (1584-1500-1584) from 5 mg (0.5% w/v) to 25 mg (2.5% w/v), a small increase in burst release occurs and shifts the best fit for release kinetics from zero order to Higuchi model while keeping a high zero order correlation $r^2$ of about 0.95 (FIG. 10).

It is possible the increase in loading dose leads to the formation of gel depots with higher amounts of insulin on the surface. Once a gel is formed in the release media, insulin molecules on depot surface dissolves leaving more pores compared to delivery systems containing less insulin. These porous structures may lead to further drug release and degradation of the matrix. Similar results were observed when PLGA/acetonitrile systems were used for the delivery of amylase (Pechenov et al., J. Control. Release, 96:149-58 (2004)) and grafted PLGA-glucose microspheres for the delivery of octreotide (Bodmer et al., J. Control. Release, 21:129-38 (1992)).
EXAMPLE 7

This example describes the conformational stability of released insulin analyzed through ultra sensitive Differential Scanning Calorimetry (DSC) (VP-DSC, MicroCal. Northampton, MA).

First, samples were centrifuged and supernatants were filtered through 0.1 µm filter. Both buffer and samples were degassed by stirring under vacuum. The heat flow required to keep the sample cell and the reference cell at the same temperature was recorded at temperature range of 25-105°C at a scan rate of 1°C/minute for insulin. To ensure that the heat transition during protein conformational changes is the only source of thermal difference between sample cell and reference cell, a baseline thermogram was obtained by loading the releasing buffer in both cells. This baseline was subtracted from sample thermogram during data analysis. Mid-point transition temperature (Tm) and the calorimetric enthalpy (ΔH) were used as conformational stability indicating thermodynamics parameters. The transition curve was fitted by non-2-state model (MN2state) which used the levenberg/ Marquardt non-linear least-square method. All data analysis was performed using Origin® software (MicroCal software, Inc., Northampton, MA) provided with the instrument.

DSC showed that the insulin released after 1 month had lower ΔH and Tm compared to fresh insulin (FIG. 11). Fresh and in vitro released insulin showed ΔH values of 14.3±0.7 and 10.1±0.5 Cal/mol x 103, Tm values of 82.49±0.92 and 83.02±0.13, respectively.

EXAMPLE 8

This example describes the secondary structure of released insulin analyzed using Circular Dichroism (CD) assays. Spectra of released insulin in the samples were recorded on a Jasco J-815 CD spectrophotometer (Jasco, Tokyo, Japan). The filtered samples were scanned in a 0.1 cm cell from 200 to 300 nm using a bandwidth of 1 nm, 0.5 second response time, 0.2 nm data pitch, and a scanning speed of 100 nm/minute. Each spectrum represents the average of three scans. Spectra of the buffer were recorded and subtracted from sample spectra. Insulin concentrations obtained by BCA assay were used to calculate the molar ellipticity [θ].

FIG. 12 represents CD spectra for fresh and the in vitro released insulin after 1 and 2 months. There was a reduction in the magnitude of both minima at 209 and 222
nm in released insulin as compared to fresh insulin. The further reduction after 2
months can be explained by additional time that released insulin remained in the
release media at 37°C. Yet, the presence of the two minima confirms that the
secondary structure of insulin was relatively conserved during delivery system
preparation and throughout release period.

EXAMPLE 9

This example further describes the study of the chemical stability of the
released insulin from smart polymer delivery systems, utilizing mass spectrometry
assays. Specifically, matrix-assisted laser desorption/ionization time-of-flight
(MALDI-TOF) mass spectrometry was utilized. Matrix solution was prepared by
dissolving 10 mg of α-cyano-4-hydroxycinnamic acid in 1 ml mixture containing 1:1
ratio of acetonitrile and 0.1% v/v TFA water solution. Ten microliters of insulin
sample were added to 100 µl of matrix solution and the solution was mixed by
vortexing. An aliquot (2 µl) of the final solution was applied to the sample target
plate and allowed to dry prior to insertion into the mass spectrometer. MALDI-TOF
experiments were carried out on a Bruker MALDI-TOF II (Bruker Daltonics Inc.,
Billercia, MA) equipped with a 200 Hz solid state smart beam laser. Samples were
run in the Positive Reflectron mode and data was analyzed using FlexAnalysis®
software provided with the instrument.

FIGS. 13 and 14 show the MALDI spectra of control fresh insulin and one
month released insulin from triblock copolymer based delivery system containing
40% (w/w) copolymer 2. The insulin primary structure integrity was conserved in the
released samples with (M+H)+ signal corresponding to a molecular mass of 5808.6
Da. No major degradation products were detected. There was no evidence of
formation of acylation products (MW+ 72) or high molecular weight transformation
(HMWT) products such as CID. However, when the released insulin spectra were
enlarged, a signal corresponding to a molecular weight of 5729.9 was observed (FIG.
15). This indicates a loss of a water molecule from the original protein which
suggests the formation of cyclic imide product that usually follows deamidation
reactions. The insulin released after two months (FIG. 15B) showed a higher peak for
the 5729 Da signal, implying that further deamidation took place. The 5729 Da signal
relative intensity percentage (compared to 5808 Da signal which corresponds to the
native insulin) was equal to 13.7% and 37.8% in released insulin samples withdrawn
after one and two months, respectively. This increase in deamidation products could be caused by the prolonged contact of insulin with the microenvironment inside the depot or the release medium. Two additional signals were observed in two months released samples corresponding to molecular weights of 5217.1 and 5070.2. These two signals appear to be products of peptide-bond hydrolysis which led to the formation of lower molecular weight peptides.

EXAMPLE 10

This example describes the in vivo absorption and bioactivity of insulin delivered with the thermo-sensitive copolymer delivery system. Both serum insulin and blood glucose levels following single subcutaneous administration of insulin-loaded temperature-sensitive delivery systems were measured.

Male Sprague-Dawley rats with body weight of 200-224g were used in the study. First, diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ; 60 mg/kg) dissolved in citrate buffer (pH = 4.5). For the first 24 hour post-injection, rats were provided with 5% sucrose solution instead of water to counteract hypoglycemia which may occur due to β-cells necrosis and endogenous insulin release. The rats were considered diabetic if fasting blood glucose level was higher than 200 mg/dl one week after STZ injection. Blood glucose level was determined using a Glucometer Elite® (Bayer Corporation, Elkhart, IN). Diabetic rats were divided into different groups (6 animals/group). The rats were anesthetized by 30 mg/kg pentobarbital sodium (Nembutal®) administered intraperitoneally.

Rats in treatment groups were injected subcutaneously at neck region with temperature-sensitive delivery systems at doses of 90 insulin unit (U)/kg body weight using 25-gauge needles. Control groups were injected with polymeric delivery systems without insulin. A solution group study was carried out by injecting insulin dissolved in PBS at a dose of 2U/kg body weight subcutaneously. Three hundred microliter blood samples were withdrawn at predetermined time points from the tail vein after an overnight fasting. Blood samples were centrifuged at 4°C and 3000 rpm for 15 minutes and serum was collected. The serum samples were frozen and stored at -20°C until further analysis. Blood glucose level was determined by the glucose oxidase method using a glucometer (Glucometer Elite®, Bayer Corporation, Elkhart, IN). AU rats were euthanized by administering pentobarbital (150 mg/kg body weight) intraperitoneally.
Serum insulin was measured by Human Insulin ELISA kit (Linco Research Inc., St. Charles, MO). Bioavailability pharmacokinetic analysis for serum insulin levels was based on the area under the curve (AUC) calculations. For the subcutaneous solution group, maximum serum concentration (C_{max}) and the time at which maximum concentration is reached (T_{max}) were determined from the concentration versus time data. Area under serum insulin concentration versus time was estimated by trapezoidal rule (Rowland and Tozer, Clinical Pharmacokinetics: Concepts and Applications, 288-289 (1980)).

FIG. 16 shows the serum insulin concentration and blood glucose level following subcutaneous administration of insulin (2 U/kg body weight) dissolved in PBS (pH=7.4) in diabetic SD rats. Serum insulin level increased rapidly reaching mean peak concentration (C_{max}) of 67.84 μU/ml at 2 hours post-administration and declined afterwards to reach below 2μU/ml (detection limit) after 12 hours. Blood glucose levels decreased following insulin absorption showing an acute and relatively short hypoglycemic effect. Blood glucose levels were restored to pre-administration levels within 6-8 hours.

FIG. 17 shows the serum insulin and blood glucose levels following single subcutaneous administration of insulin-loaded temperature-sensitive delivery systems. Treatment groups included insulin-loaded (90 U/kg body weight) dissolved in 40% (w/w) of either copolymer 1 (1496-1500-1496) or copolymer 2 (1584-1500-1584). Zinc was added to both treatment groups as zinc acetate (1:5 ratio). Control group showed very low or undetectable concentrations while treatment groups showed continuous insulin release from thermo-sensitive delivery systems over a period of three months. The more hydrophobic copolymer 2 showed a lower release rate for longer duration compared to copolymer 1. Blood glucose levels from both delivery systems were significantly lower than the diabetic rats control group. Mean blood glucose levels were below 200 mg/dl in rats injected with copolymer 2 delivery systems over a month.

The bioavailability of insulin in rats was calculated in terms of AUC values after correcting for dose. TABLE 6 shows the in vivo pharmacokinetic parameters of insulin delivery systems in rats. When compared to subcutaneous insulin solution, temperature-sensitive based delivery systems enhanced insulin bioavailability by 7.59-10.91 fold. Increasing the PLA chain length in PLA-PEG-PLA triblock thermo-sensitive delivery systems increased insulin bioavailability.
TABLE 6.

<table>
<thead>
<tr>
<th>Group</th>
<th>$C_{max}$ (µU/ml)</th>
<th>$T_{max}$ (h)</th>
<th>AUC (µU.h/ml)</th>
<th>Increase in AUC (folds compared to S.C.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.C.</td>
<td>67.84 ± 7.38</td>
<td>1.00</td>
<td>5493.83 ± 1263.72</td>
<td>-</td>
</tr>
<tr>
<td>T1</td>
<td>-</td>
<td>-</td>
<td>41709.90 ± 8116.30$^a$</td>
<td>7.59</td>
</tr>
<tr>
<td>T2</td>
<td>-</td>
<td>-</td>
<td>59924.41 ± 11279.68$^a$</td>
<td>10.91</td>
</tr>
</tbody>
</table>

S.G.: Insulin solution group.

T1: Thermo-sensitive delivery systems - copolymer 1 (1496-1500-1496).

T2: Thermo-sensitive delivery systems - copolymer 2 (1584-1500-1584).

$^a$ Significantly (p < 0.05) higher than solution group.

The ultimate success of insulin controlled delivery systems is the ability to control the in vivo release and lower blood glucose level for longer duration. In the study, diabetic model was induced by injecting STZ into male SD rats intraperitoneally. STZ is transported to pancreatic β cells via glucose transporter GLUT2 (Thulesen et al., Endocrinology, 138:62-8 (1997)) where it induces cell death by alkylation of DNA due to its nitrosourea moiety (Eisner et al., Diabetologia, 43:1528-33 (2000)). Insulin solution group (dissolved in PBS, pH = 7.4) showed a sharp increase in serum insulin level following subcutaneous administration accompanied by a parallel reduction in blood glucose levels. Insulin and glucose levels were restored to pre-administration levels within 6-8 hours.

Thermo-sensitive delivery systems based on copolymers 1 and 2 controlled the release of insulin over three months period and corresponding reduction in blood glucose levels was observed. There was a slight increase in the duration of insulin release and blood glucose levels reduction in delivery systems containing copolymer 2 brought about by the increase in PLA chain which caused a slower degradation. In vivo pharmacokinetic parameters show that the delivery systems of the present invention enhanced insulin bioavailability compared to subcutaneous insulin solution as denoted by the increase in AUC.

Insulin absorption rates from subcutaneous tissue are inversely correlated to insulin concentration (Lauritzen et al., Horm. Metab. Res., 16:61 1-2 (1984); Chantelau et al., Diabete. Metab., 11:106-10 (1985)). The slower absorption rate leads to a higher percentage of insulin degradation by proteases in skin (Lee, Crit.
However, controlled delivery systems, such as the various embodiments of the present invention, release small amounts of insulin at a given time leading to higher absorption rates.

EXAMPLE 11

This example describes the *in vitro* biocompatibility of the insulin delivery systems of the present invention and its degradation products.

Polymeric delivery systems were extracted in PBS (pH 7.4) by keeping samples of gel depot for 10 days at 37°C and 70°C (USP 27, 1031:2404-6 (2004)). The latter group demonstrates the faster polymer degradation at elevated temperatures, simulating the long-term effects of in situ depot degradation (Rozema et al., *Degradation Phenomena of Polymeric Biomaterials*, 123-31 (1992)). After 10 days, the pH of extracts was measured and adjusted to 7.4 by adding 1M NaOH to eliminate the effect of pH on cell growth. The extracts were then filtered and diluted with growth medium to a ratio series of 1:1 through 1:16.

**MTT assay** is based on the ability of living cells to reduce a water-soluble yellow dye, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide to a water-insoluble purple formazan product by mitochondrial succinate dehydrogenase. The insoluble crystals are solubilized by the addition of a suitable solvent and absorbance is measured. The intensity of the color is related to the number of viable cells. The cells used for the assay were Human Embryonic Kidney (HEK293) cells.

Growth medium (without the addition of polymer extract) was used as negative control. HEK293 cells (8x10³ per well) were plated into 96-well microtiter plates and incubated for 48 hours to allow for cell attachment. One hundred microliters per well of a freshly prepared dilution series of the extracts were added. Eight replicates were prepared for each dilution and for the negative control. The plates were incubated for 24, 48, and 72 hours at 37°C in a humidified 5% CO₂ atmosphere. Twenty microliters of MTT solution (5 mg/ml PBS, pH 7.4) was added per well and incubated for 4 hours. Two hundred microliters per well of DMSO was used to dissolve the formed formazan crystals. The colorimetric staining of the plates was read by MRX-Microplate Reader at 570 nm.

FIG. 18 and FIG. 19 show the MTT cell viability assay for thermo-sensitive delivery systems extracts prepared from copolymer 1 and copolymer 2, respectively.

No significant difference (p > 0.05) in the cell viability was observed between
polymers extracts groups and PBS groups for all the dilutions and exposure time points (24, 48, and 72 hours). *In vitro* biocompatibility could vary fundamentally by altering cell lines, exposure time, and growth media type. Therefore, the response of tissue to which the delivery system is applied is required to test for possible signs of toxicity.

This data is significant, since although it is known that PLA-based polymers biodegrade into lactic acid, which enters the tricarboxylic acid cycle to be metabolized and eliminated from the body as carbon dioxide and water (Tice and Cowser, *Pharmcol. Technol.*, 11:26-35 (1984)), the biocompatibility of polyesters remains controversial. Studies have yielded conflicting results; certain studies describing the good biocompatibility of polyesters (Royals et al., *J. Biomed. Mater. Res.*, 45:231-9 (1999); Kranz et al., *Int. J. Pharm.*, 212:1 1-8 (2001)), where further studies report deleterious tissue responses and osteolytic reactions (Ismail et al., *Pharm. Dev. Technol.*, 5:391-7 (2000); De Jong et al., *Biomaterials*, 26:1781-91 (2005)).

**EXAMPLE 12**

This example further describes studies relating to the *in vivo* biocompatibility of the insulin delivery systems of the present invention. The *in vivo* biocompatibility of polymeric delivery systems is essential for its utility in clinical applications. Although a number of PLA-containing systems have been approved by the FDA for drug delivery (Royals et al., *J. Biomed. Mater. Res.*, 45:231-9 (1999)), the response of tissue to polymeric implants depends on many factors; including, amount of polymer, nature of degradation products, rate of degradation, and degradation products accumulation in the tissue.

Expected tissue reactions to polymeric implants are short-term inflammatory response to injury with minimal fibrosis resulting from wound healing process (Ziats et al., *Biomaterials*, 9:5-13 (1987)). Tissue response to injected implants often occurs in phases (Anderson and Shive, *Adv. Drug. Deliv. Rev.*, 28:5-24 (1997)). Often the first phase takes place within the first two weeks following injection and is characterized by the initiation and resolution of acute and chronic inflammatory responses, requiring the activation of neutrophils, lymphocytes, and monocytes. The second phase involves monocytes migrating to the injury site and differentiating into macrophages which may combine to form foreign body giant cells (FBGC). The
length of time of the second phase depends on degradation rate and ability of macrophages, and if necessary FBGCs, to clear the tissue.

The biocompatibility of the polymeric delivery systems was evaluated by studying rats’ skin tissue for inflammatory changes after administration of the polymeric delivery systems. One hundred µl of formulation was injected subcutaneously into the upper portion of neck of the rat where a visible gel lump was formed. At different time points (days 1, 7, 30, and 90), rats were euthanized and skin tissue from injection sites were surgically removed. Skin samples were then fixed in 10% neutrally buffered formalin solution. After collection of the skin samples, they were washed with water to remove excess fixative, dehydrated by transferring to increasing strengths of alcohol, and embedded in paraffin. Transverse sections of 5 µm thick were cut by rotatory microtome, mounted on a glass slide, and stained with hematoxylin and eosin. The slides were observed under light microscopy for the presence of any signs of acute and chronic inflammation, fibrous capsule formation, fibrosis, tissue morphology, and necrosis.

The inflammatory response of rat skin to temperature-sensitive delivery systems was evaluated at different time points after subcutaneous administration. Following administration, inflammatory cells (mainly neutrophils and lymphocytes) infiltrate to injection site. Within a week, lymphocytes count dropped and an abundance of macrophages, which are components of chronic tissue inflammation in response to injury (Anderson and Shive, Adv. Drug. Deliv. Rev., 28:5-24 (1997)), was observed. Skin samples from three months time point were comparable to control tissue.

The connective and muscular tissues of the delivery systems injection sites appeared normal and comparable to control skin tissue. No signs of fibrosis, muscle damage, or necrosis were observed throughout the studies denoting the biocompatible nature of the delivery systems. Histological evaluation of skin samples retrieved from injection sites of polymeric delivery systems showed a typical response to injury and normal wound healing process. Acute inflammatory response was obvious in day 1 samples indicated by the infiltration of neutrophils and lymphocytes. After one week, there was a noticeable reduction in inflammatory cells due to the resolution of acute response. Large numbers of macrophages were observed which indicate chronic inflammatory response to injury incidence. Less inflammatory cells were identified
one month post injection signifying the subsiding of the inflammatory response. All
signs of inflammatory responses diminished after 3 months, in accordance with in
vitro MTT assay results and ISO regulations, demonstrating the biocompatibility of
the polymeric delivery system.

All publications and patent applications in this specification are indicative of
the level of ordinary skill in the art to which this invention pertains. All publications
and patent applications are herein incorporated by reference to the same extent as if
each individual publication or patent application was specifically and individually
indicated by reference.
What is claimed is:

1. A thermo-sensitive controlled drug delivery system capable of releasing a drug over a longer period of time comprising an injectable biodegradable and biocompatible polylactic acid-polyethylene glycol-polylactic acid (PLA-PEG-PLA) triblock polymer, wherein said triblock polymer concentration is from about 25% to 40%; and a biologically active agent released at zero order kinetics.

2. The delivery system of claim 1 wherein the average molecular weight of said triblock polymer is 4991 to 7000.

3. The delivery system of claim 2 wherein the average molecular weight of said triblock polymer is 4991 to 6100.

4. The delivery system of claim 1 wherein the polylactic acid has a chain length of from about 800 to 2300 Da and said polyethylene glycol has a chain length of from about 400 to 1500 Da.

5. The delivery system of claim 4 wherein the polylactic acid has a chain length of from about 1400 to 1600 Da and the polyethylene glycol has a chain length of about 1500 Da.

6. The delivery system of claim 1 wherein the polylactic acid-polyethylene glycol-polylactic acid (PLA-PEG-PLA) triblock polymer chain has a length of about 1496-1500-1496 Da.

7. The delivery system of claim 1 wherein the polylactic acid-polyethylene glycol-polylactic acid (PLA-PEG-PLA) triblock polymer chain has a length of from about 1584-1500-1584 Da.

8. The delivery system of claim 5 wherein said triblock polymer concentration is from about 30% to 40%.
9. The delivery system of claim 8 wherein said triblock polymer is a solution at
room temperature and is a gel at approximately 37°C.

10. The delivery system of claim 9 wherein said biologically active agent is
selected from the group consisting of a peptide, protein, hormone or
combination of the same.

11. The delivery system of claim 10 wherein the protein is insulin.

12. The delivery system of claim 11 wherein the insulin comprises from about
0.5% to 9% of the w/w% of the delivery system.

13. The delivery system of claim 12 wherein the insulin comprises from about
4.5% to 9% of the w/w% of the delivery system.

14. The delivery system of claim 13 wherein said insulin is released for a period
of from about two to three months.

15. A triblock copolymer capable of releasing insulin over a sustained period of
time comprising a biocompatible and biodegradable thermo-sensitive plurality
of polylactic acid-polyethylene glycol-polylactic acid (PLA-PEG-PLA)
polymers.

16. A vehicle for delivery of a biologically active agent over a period of two to
three months comprising polylactic acid-polyethylene glycol-polylactic acid
(PLA-PEG-PLA) triblock polymers, wherein said polylactic acid has a chain
length of about 1500 Da and said polyethylene glycol has a chain length of
from about 1496 to 1584 Da; a biologically active agent; and an aqueous
solution.

17. The vehicle of claim 16 wherein the aqueous system is injectable and thermo-
sensitive, wherein said vesicle is capable of transitioning from an aqueous
solution to a gel depot form upon injection to a warm-blooded patient in need
of the biologically active agent.
18. The vehicle of claim 17 wherein the aqueous system is biodegradable and biocompatible.

19. The vehicle of claim 18 wherein the triblock polymer has a chain length of from about 1496-1500-1496 Da.

20. The vehicle of claim 18 wherein the triblock polymer has a chain length of from about 1584-1500-1584 Da.

21. The vehicle of claims 19 or 20 wherein the biologically active agent is insulin.

22. The vehicle of claim 21 further comprising zinc ions in a ratio of 1:5 (insulin to zinc).

23. A method of treating diabetes comprising: administering to a patient in need thereof the drug delivery system of claim 12.

24. The method of claim 23 wherein said insulin is released for a period of two to three months at a constant rate of 0.5 to 1 unit of insulin per hour.

25. The method of claim 23 further comprising injecting said patient with said insulin delivery system from about every two to three months.
FIG. 1
FIG. 2

EG (Ch2)
PLA (CH3)
PLA (CH)
PLA (OH)

ppm

Auto-Scaled Chromatogram

MV

Minutes

0.00  2.00  4.00  6.00  8.00  10.00  12.00  14.00  16.00  18.00  20.00  22.00  24.00

0.00  2.00  4.00  6.00  8.00  10.00  12.00

6380

FIG. 3

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<th>Retention Time</th>
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FIG. 4

FIG. 5
FIGS. 16A & 16B
FIGS. 18A & 18B
**FIGS. 19A & 19B**