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- (54) **Title:** NOVEL LONG-ACTING INSULIN ANALOGUE AND USE THEREOF

- (57) **Abstract:** The present invention relates to an insulin analogue conjugate having a reduced insulin receptor binding affinity for increased in-vivo duration and bioavailability of insulin, and a long-acting insulin formulation using the same, a long-acting formulation for preventing or treating diabetes including the conjugate, and a method for treating diabetes using the conjugate, the long-acting insulin formulation, or the long-acting formulation for preventing or treating diabetes.



Description

Title of Invention: NOVEL LONG-ACTING INSULIN ANALOGUE AND USE THEREOF

Technical Field

- [1] The present invention relates to an insulin analogue conjugate having a reduced insulin receptor binding affinity for increased in-vivo duration and bioavailability of insulin, and a long-acting insulin formulation using the same, a long-acting formulation for preventing or treating diabetes including the conjugate, and a method for treating diabetes using the conjugate, the long-acting insulin formulation, or the long-acting formulation for preventing or treating diabetes.

[2]

Background Art

- [3] Insulin is a hormone secreted by the pancreas of the human body, which regulates blood glucose levels, and plays a role in maintaining normal blood glucose levels while carrying surplus glucose in the blood to cells to provide energy for cells. In diabetic patients, however, insulin does not function properly due to lack of insulin, resistance to insulin, and loss of beta-cell function, and thus glucose in the blood cannot be utilized as an energy source and the blood glucose level is elevated, leading to hyperglycemia. Eventually, urinary excretion occurs, contributing to development of various complications. Therefore, insulin therapy is essential for patients with abnormal insulin secretion (Type I) or insulin resistance (Type II), and blood glucose levels can be normally regulated by insulin administration. Like other protein and peptide hormones, insulin has a very short in vivo half-life, and thus has a disadvantage of repeated administration.
- [4] Such frequent administration causes severe pain and discomfort for the patients. For this reason, in order to improve quality of life by increasing in vivo half-life of the protein and reducing the administration frequency, many studies on protein formulation and chemical conjugation (a fatty acid conjugate, a polyethylene polymer conjugate) have been conducted. Commercially available long-acting insulin includes insulin glargine manufactured by Sanofi Aventis (lantus, lasting for about 20-22 hours), and insulin detemir (levemir, lasting for about 18-22 hours) and tresiba (degludec, lasting for about 40 hours) manufactured by Novo Nordisk. These long-acting insulin formulations produce no peak in the blood insulin concentration, and thus they are suitable as basal insulin. However, because these formulations do not have sufficiently long half-life, the disadvantage of one or two injections per day still remains. Accordingly, there is a limitation in achieving the intended goal that admin-

istration frequency is remarkably reduced to improve convenience of diabetic patients in need of long-term administration.

- [5] In-vivo insulin clearance process is reported in Biochem J (1998) 332, 421, Endocrine Reviews (1998) 19, 608, J Am Col Nut (2003) 22, 487; 50% or more of insulin is removed in the kidney and the rest is removed via a receptor-mediated clearance (RMC) process in target sites such as muscle, fat, liver, etc.
- [6] In this regard, many studies, including J Pharmacol Exp Ther (1998) 286: 959, Diabetes Care (1990) 13: 923, Diabetes (1990) 39: 1033, have reported that in-vitro activity is reduced to avoid RMC of insulin, thereby increasing the blood level. J. Biol. Chem.(1997) 83: 12978 and Biomed. Res. (1984) 5: 267 have reported that insulin receptor binding affinity is reduced by mutation of insulin amino acids.
- [7] However, these insulin analogues having reduced receptor binding affinity cannot avoid renal clearance which is a main clearance mechanism, although RMC is reduced. Accordingly, there has been a limit in remarkably increasing the blood half-life.

[8]

Disclosure of Invention

Technical Problem

- [9] The present inventors have made many efforts to increase the blood half-life of insulin. As a result, they have found that when a conjugate using an insulin analogue having a reduced insulin receptor binding affinity and reduced receptor mediated clearance (RMC) of insulin is used as a formulation capable of increasing half-life and bioavailability or maintaining its activity, the blood half-life of insulin is further increased, thereby completing the present invention.

[10]

Solution to Problem

- [11] An object of the present invention is to provide an insulin analogue conjugate which is formed by linking a novel insulin analogue having a reduced insulin receptor binding affinity with polyethylene glycol, an amino acid polymer, or a combination thereof, for the purpose of prolonging in-vivo half-life of insulin.
- [12] Another object of the present invention is to provide a long-acting insulin formulation including the insulin analogue conjugate, in which the formulation has increased in-vivo duration and stability.
- [13] Still another object of the present invention is to provide a long-acting formulation for preventing or treating diabetes, including the insulin analogue conjugate.
- [14] Still another object of the present invention is to provide a method for treating insulin-related diseases, including the step of administering the insulin analogue conjugate, the long-acting insulin formulation, or the long-acting formulation for

preventing or treating diabetes to a subject in need thereof.

Advantageous Effects of Invention

- [15] A long-acting insulin analogue conjugate of the present invention is a conjugate, in which an analogue having a reduced insulin receptor binding affinity, compared to the native form, is linked with a carrier having increased blood half-life, and it is used to provide a long-acting insulin formulation capable of increasing in-vivo half-life and bioavailability, and/or maintaining activity. Accordingly, it may be used to treat a variety of insulin-related diseases while improving patients' convenience.

[16]

Best Mode for Carrying out the Invention

- [17] In order to achieve the above objects, an aspect of the present invention provides an insulin analogue conjugate having a reduced insulin receptor binding affinity, compared to a native insulin.

- [18] Specifically, the insulin analogue conjugate may be an insulin analogue conjugate having a reduced insulin receptor binding affinity, compared to the native insulin, in which the conjugate has the following Chemical Formula:

[19]

[20] [Chemical Formula]

[21] X-La-F

- [22] wherein X is an insulin analogue having a reduced insulin receptor binding affinity, compared to the native insulin,

[23]

- [24] L is a linker, and a is 0 or a natural number (if a is 2 or larger, each L is independent), and

- [25] F is polyethylene glycol, an amino acid polymer, or a combination thereof.

[26]

- [27] The insulin analogue conjugate of the present invention is found to have a new structure, in which RMC is avoided due to the reduced insulin receptor conjugate, and the insulin receptor binding affinity of the insulin analogue is reduced despite binding of the linker or carrier when a biocompatible material F binds thereto as a carrier, and the insulin receptor binding affinity of the insulin analogue is reduced to avoid RMC without affecting the function of insulin itself, thereby increasing in-vivo half-life and bioavailability, and/or maintaining its activity, and in-vivo half-life of polyethylene glycol and/or amino acid polymer linked to the insulin analogue is remarkably increased, leading to providing the insulin analogue conjugate.

[28]

- [29] As used herein, the term "insulin analogue" refers to a substance that is obtained by

mutating one or more amino acids in the native insulin sequence.

- [30] The mutation of one or more amino acids in the native sequence refers to that an alteration selected from the group consisting of substitution, addition, deletion, modification and combinations thereof occurs in one or more amino acids of native insulin.
- [31] The insulin analogue may be an insulin analogue having reduced insulin titer and/or reduced insulin receptor binding affinity, compared to the native form, in which an amino acid of B chain or A chain of insulin is mutated or deleted. Any amino acid analogue having reduced insulin titer and/or reduced insulin receptor binding affinity, compared to the native form, may be included without limitation.
- [32] The amino acid sequences of the native insulin are as follows.
- [33]
- [34] A chain:
- [35] Gly-Ile-Val-Glu-Gln-Cys-Cys-Thr-Ser-Ile-Cys-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn (SEQ ID NO: 61)
- [36] B chain:
- [37] Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr (SEQ ID NO: 62)
- [38]
- [39] The insulin used in embodiments of the present invention may be an insulin analogue produced by a genetic recombination technology, but the present invention is not limited thereto. The insulin includes all insulins having reduced in-vitro titer and/or reduced insulin receptor-binding affinity. Preferably, the insulin includes inverted insulin, insulin variants, insulin fragments, etc., and it may be prepared by a solid phase method as well as a genetic recombination method, but is not limited thereto.
- [40] The insulin analogue is a peptide that retains the function of controlling the blood glucose level in the body, which is equal to that of insulin, and such peptide includes agonists, derivatives, fragments, and variants of insulin.
- [41]
- [42] The insulin agonist of the present invention refers to a compound that binds to the insulin receptor to show the biological activity equal to that of insulin, which is irrelevant to the structure of insulin.
- [43] The insulin analogue of the present invention refers to a peptide having a homology with respective amino acid sequences of A chain and B chain of the native insulin, which may have at least one amino acid residue mutated by an alteration selected from the group consisting of substitution (e.g., alpha-methylation, alpha-hydroxylation), deletion (e.g., deamination), modification (e.g., N-methylation), and combinations thereof, and has a function of regulating the blood glucose level in the body.
- [44] In the present invention, the insulin analogue refers to a peptide mimic or a low- or

high-molecular-weight compound that binds to the insulin receptor to regulate the blood glucose level, even though its amino acid sequence has no homology with that of the native insulin.

[45] The insulin fragment of the present invention refers to a fragment having one or more amino acids added or deleted at insulin, in which the added amino acids may be non-naturally occurring amino acids (e.g., a D-type amino acid), and this insulin fragment has a function of regulating the blood glucose level in the body.

[46] The insulin variant of the present invention is a peptide having one or more amino acid sequences different from those of insulin, and it refers to a peptide that retains the function of regulating the blood glucose level in the body.

[47] Each of the preparation methods for the insulin agonists, derivatives, fragments, and variants of the present invention may be used individually or in combination. For example, the present invention includes a peptide that has one or more different amino acids and deamination of the N-terminal amino acid residue, and has a function of regulating the blood glucose level in the body.

[48]

[49] Specifically, the insulin analogue may be prepared by substitution of one or more selected from the group consisting of amino acid residues at positions 1, 2, 3, 5, 8, 10, 12, 16, 23, 24, 25, 26, 27, 28, 29, and 30 of B chain and at positions 1, 2, 5, 8, 10, 12, 14, 16, 17, 18, 19 and 21 of A chain with other amino acid(s). Examples of other amino acids to be substituted may include alanine, glutamic acid, asparagine, isoleucine, valine, glutamine, glycine, lysine, histidine, cysteine, phenylalanine, tryptophan, proline, serine, threonine, and aspartic acid, but any substitution capable of causing a reduction in the insulin receptor binding affinity may be included without limitation. Further, an insulin analogue in which one or more amino acids are deleted to reduce the insulin receptor binding affinity may be also included in the scope of the present invention, but there is no limitation in the insulin analogue having reduced insulin receptor binding affinity.

[50]

[51] The insulin analogue conjugate of the present invention is characterized in that it has a long-acting property capable of increasing in-vivo half-life and/or bioavailability, and/or maintaining its activity.

[52] The present invention confirmed that the insulin analogue is applied to a formulation capable of increasing half-life and bioavailability of the insulin analogue and/or maintaining its activity, thereby remarkably increasing its half-life and in-vivo activity, compared to the native insulin.

[53]

[54] In the present invention, F of the insulin analogue conjugate may be a biocompatible

material or carrier capable of increasing half-life and bioavailability of the insulin analogue or maintaining its activity, and there is no limitation in the constitution. Example thereof may include polyethylene glycol or an amino acid polymer, or a combination thereof, but is not limited thereto.

[55] As used herein, the term "biocompatible material or carrier" refers to a substance capable of increasing in-vivo half-life of the insulin analogue to prolong duration of its activity when it is linked to the insulin analogue via a covalent or non-covalent bond to form a conjugate. Since the primary goal of the present invention is to increase half-life and bioavailability and to maintain the activity of the insulin analogue, the biocompatible material to be linked to the insulin analogue having reduced insulin receptor binding affinity may include various biocompatible materials, e.g., polyethylene glycol, fatty acid, cholesterol, albumin and a fragment thereof, an albumin-binding material, elastin, a water-soluble precursor of elastin, and a polymer of repeating units of a part of the elastin protein sequence, a polymer of repeating units of a particular amino acid sequence, an antibody, an antibody fragment, FcRn binding material, connective tissues, nucleotides, fibronectin, transferrin, saccharides, polymers, etc., without limitation. Further, the linkage method between the insulin analogue having the reduced insulin receptor binding affinity and the biocompatible material capable of prolonging in-vivo half-life includes genetic recombination and in vitro conjugation using a high- or low-molecular-weight compound, but is not limited to any linkage method. The FcRn binding material may be an immunoglobulin Fc region.

[56] When polyethylene glycol (PEG) is used as a carrier, Ambrx' ReCODE technology capable of site-specifically attaching polyethylene glycol may be employed, and Neose's glycopegylation technology capable of sugar chain-specifically attaching polyethylene glycol may be employed. Further, releasable PEG technology of slowly eliminating polyethylene glycol in the body may be also employed, but is not limited thereto. It is apparent that any technology capable of increasing bioavailability using PEG may be employed. Further, polymers such as polyethylene glycol, polypropylene glycol, an ethylene glycol-propylene glycol copolymer, polyoxyethylated polyol, polyvinyl alcohol, polysaccharide, dextran, polyvinyl ethyl ether, a biodegradable polymer, a lipid polymer, chitin, or hyaluronic acid may be also included.

[57] When albumin is used as a carrier, a technology of directly linking albumin or an albumin fragment to the insulin analogue by a covalent bond to increase in-vivo stability may be employed. When albumin is not directly linked, a technology of linking an albumin-binding material, for example, an albumin-specific antibody or antibody fragment to the insulin analogue and then linking it to albumin, a technology of linking a particular peptide/protein having albumin-binding affinity to the insulin

analogue, and a technology of linking fatty acids having albumin-binding affinity may be employed, but is not limited thereto. Any technology or linkage method capable of increasing in-vivo stability using albumin may be employed.

[58] A technology of linking an antibody or antibody fragment as a carrier to the insulin analogue in order to increase in-vivo half-life may be also included in the present invention. The antibody or antibody fragment may be an antibody or antibody fragment having an FcRn-binding site, and it may be any antibody fragment containing no FcRn-binding site such as Fab, etc. CovX's CovX-body technology of using a catalytic antibody may be employed, and it is apparent that a technology capable of increasing in-vivo half-life using an Fc fragment may be also employed in the present invention. When the Fc fragment is used, a linker binding to the Fc fragment and the insulin analogue, or a linkage method may be polyethylene glycol or a peptide bond, etc., but is not limited thereto. Any chemical linkage may be employed. Further, a binding ratio of the Fc fragment and insulin analogue may be 1:1 or 1:2, but is not limited thereto.

[59] A technology of linking a peptide or protein fragment as a carrier to the insulin analogue in order to increase in-vivo half-life may be also included in the present invention. The peptide or protein fragment to be used may include Elastin-like polypeptide (ELP) composed of repeating units of a particular amino acid combination, and Xten technology of Versatis Inc. using an artificial polypeptide PEG may also be included in the present invention. Zealand's Structure inducing probe (SIP) technology of increasing in-vivo half-life using multi-Lysine may also be employed, and Prolor's CTP fusion technology may also be employed. Use of transferrin which is known to increase in-vivo stability, fibronectin, which is a connective tissue component, or derivatives thereof may be also employed. The peptide or protein binding to the insulin analogue is not limited thereto, and any peptide or protein capable of increasing in-vivo half-life of the insulin analogue may also be included in the scope of the present invention. Further, linkage between the insulin analogue and the peptide or protein may be a covalent bond, the kind of the linker may be polyethylene glycol, or the linkage method may be a peptide bond, but is not limited thereto. Any chemical linkage method may be used.

[60] Further, the carrier used to increase in-vivo half-life may be a non-peptide material such as polysaccharide, fatty acid, etc.

[61] The polyethylene glycol may be in a linear or branched form, and it means any polyethylene glycol that can be attached to the insulin analogue showing reduced insulin RMC (receptor-mediated clearance), compared to the native form and there is no limitation in its size, as long as it is within the range of 1 Da ~ 100 kDa, or 10 Da ~ 100 kDa. Further, one or more of polyethylene glycol may be linked thereto.

- [62] The polyethylene glycol may be linked to one or more amino acid residues selected from the group consisting of the N-terminal amino acid and the C-terminal amino acid of the insulin analogue, and lysine, cysteine, aspartic acid, and glutamic acid within the insulin analogue, but is not limited thereto.
- [63] A particular amino acid sequence may be an amino acid polymer capable of increasing in-vivo half-life when it is linked to the insulin analogue showing reduced insulin RMC (receptor-mediated clearance), and it may be a protein constituting the connective tissue in the body such as elastin, but is not limited thereto. Further, it may be a non-naturally occurring amino acid polymer by artificial combination of a certain repeating unit of elastin. Elastin may be human tropoelastin (SEQ ID NO: 48), which is a water-soluble precursor, and a polymer of a partial sequence thereof or repeating units thereof may be also included.
- [64] For example, the amino acid sequence may be a repeated sequence of a repeating unit (VPGXG, SEQ ID NO: 49)_n, wherein X may be selected from alanine, arginine, asparagine, aspartic acid, glutamic acid, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine residues, and n may be 1 to 500.
- [65]
- [66] Specifically, the repeating unit may be an amino acid polymer selected from (VPGVG, SEQ ID NO: 50)_n, (VPGAG, SEQ ID NO: 51)_n, (VPGGG, SEQ ID NO: 52)_n, (VPGIG, SEQ ID NO: 53)_n, (VPGLG, SEQ ID NO: 54)_n, (VPGFG, SEQ ID NO: 55)_n, (AVGVVP, SEQ ID NO: 56)_n, (IPGVG, SEQ ID NO: 57)_n, and (LPGVG, SEQ ID NO: 58)_n, or an elastin-like peptide (ELP) which is a polymer obtained from combination of the repeating units, but is not limited thereto. A polymer composed of repeating units of any sequence may be also included in the scope of the present invention (n=1~500).
- [67] Abbreviations of the amino acids used herein are in accordance with the IUPAC-IUB nomenclature as follows.
- [68] Alanine A Arginine R
- [69] Asparagine N Aspartic acid D
- [70] Cysteine C Glutamic acid E
- [71] Glutamine Q Glycine G
- [72] Histidine H Isoleucine I
- [73] Leucine L Lysine K
- [74] Methionine M Phenylalanine F
- [75] Proline P Serine S
- [76] Threonine T Tryptophan W
- [77] Tyrosine Y Valine V
- [78]

- [79] Elastin, a water-soluble precursor of elastin, and an elastin-like polypeptide may be obtained from native forms that are isolated from humans and other animals including cows, goats, swine, mice, rabbits, hamsters, rats, and guinea pigs, or may be recombinant forms obtained from transformed animal cells or microorganisms. Further, addition of sugar chain may be performed by adding sugar chain-modified amino acids during production in animal cells.
- [80] In the present invention, binding of elastin, the water-soluble precursor of elastin or the elastin-like polypeptide to insulin showing reduced insulin receptor-mediated clearance (RMC) may be performed by a peptide bond via genetic recombination, or they may be produced separately and then bind to each other by a non-peptidyl polymer.
- [81]
- [82]
- [83] The linker, which is able to link between the insulin analogue having reduced insulin receptor-binding affinity and the carrier capable of increasing in-vivo half-life of the insulin analogue, may be a peptide or a non-peptidyl polymer.
- [84] The non-peptidyl polymer may be formed by any chemical bond such as a non-covalent chemical bond or a covalent chemical bond, but there is no limitation thereto.
- [85] The non-peptidyl polymer means a biocompatible polymer formed by linking two or more of repeating units, and the repeating units are linked to each other via not a peptide bond but any covalent bond. Such non-peptidyl polymer may have two or three ends.
- [86] The non-peptidyl polymer useful in the present invention may be selected from the group consisting of polyethylene glycol, fatty acids, saccharides, polymers, low-molecular-weight compounds, nucleotides, and combinations thereof.
- [87] The polymer may be selected from the group consisting of a biodegradable polymer such as polyethylene glycol, polypropylene glycol, an ethylene glycol-propylene glycol copolymer, polyoxyethylated polyol, polyvinyl alcohol, polysaccharide, dextran, polyvinyl ethyl ether, polylactic acid (PLA) and polylactic-glycolic acid (PLGA), a lipid polymer, chitin, hyaluronic acid, an oligonucleotide, and combinations thereof. Specifically, the polymer may be polyethylene glycol, but is not limited thereto. Derivatives thereof known in the art and derivatives easily prepared by a method known in the art may be included in the scope of the present invention.
- [88] The peptidyl linker used in the fusion protein obtained by a conventional inframe fusion method has drawbacks that it is easily cleaved in vivo by a proteolytic enzyme, and thus a sufficient effect of increasing the serum half-life of the active drug by a carrier cannot be obtained as expected. In the present invention, however, a non-peptidyl linker as well as the peptidyl linker may be used to prepare the conjugate. In

the non-peptidyl linker, a polymer having resistance to the proteolytic enzyme may be used to maintain the serum half-life of the peptide being similar to that of the carrier. Therefore, any non-peptidyl polymer can be used without limitation, as long as it is a polymer having the aforementioned function, that is, a polymer having resistance to the in-vivo proteolytic enzyme. The non-peptidyl polymer has a molecular weight in the range of 1 to 100 kDa, and preferably of 1 to 20 kDa.

- [89] Polyethylene glycol or elastin-like peptide, which is a carrier linked via the non-peptidyl polymer of the present invention, may be a single type of carrier or a combination of different types of carriers by the non-peptidyl polymer.
- [90] The non-peptidyl polymer used in the present invention has a reactive group capable of binding to an elastin-like peptide and a protein drug.
- [91] The non-peptidyl polymer has a reactive group at both ends, which is preferably selected from the group consisting of a reactive aldehyde group, a propionaldehyde group, a butyraldehyde group, a maleimide group and a succinimide derivative. The succinimide derivative may be succinimidyl propionate, hydroxy succinimidyl, succinimidyl carboxymethyl, or succinimidyl carbonate. In particular, when the non-peptidyl polymer has a reactive aldehyde group at both ends thereof, it is effective in linking at both ends of the non-peptidyl polymer with a physiologically active polypeptide and an immunoglobulin with minimal non-specific reactions. A final product generated by reductive alkylation by an aldehyde bond is much more stable than that linked by an amide bond. The aldehyde reactive group selectively binds to an N-terminus at a low pH, and binds to a lysine residue to form a covalent bond at a high pH, such as pH 9.0.
- [92] The reactive groups at both ends of the non-peptidyl polymer may be the same as or different from each other. For example, the non-peptidyl polymer may possess a maleimide group at one end, and an aldehyde group, a propionaldehyde group or a butyraldehyde group at the other end. When polyethylene glycol having a reactive hydroxy group at both ends thereof is used as the non-peptidyl polymer, the hydroxy group may be activated to various reactive groups by known chemical reactions, or a polyethylene glycol having a commercially available modified reactive group may be used so as to prepare the single-chain insulin analogue conjugate of the present invention.
- [93] Such insulin analogue conjugate of the present invention enables to maintain in-vivo activities of the conventional insulin, such as energy metabolism and sugar metabolism, and also enables to increase blood half-life of the insulin analogue and markedly increase duration of in-vivo efficacies of the peptide, and therefore, the conjugate is useful in the treatment of diabetes.

[94]

- [95] In an embodiment of the present invention, it was confirmed that the insulin analogue having a reduced insulin receptor binding affinity exhibits a much higher in-vivo half-life than the native insulin conjugate, when the analogue is linked to the carrier capable of prolonging in-vivo half-life.
- [96]
- [97] In another aspect, the present invention provides a long-acting insulin formulation including the insulin analogue conjugate. The long-acting insulin formulation may be a long-acting insulin formulation having increased in-vivo duration and stability. The long-acting formulation may be a pharmaceutical composition for treating diabetes.
- [98] In still another aspect, the present invention provides a long-acting insulin formulation for preventing or treating diabetes, including the insulin analogue conjugate.
- [99]
- [100] An agent capable of increasing bioavailability or maintaining activity may include a sustained release formulation by microparticles or nanoparticles using PLGA, hyaluronic acid, chitosan, etc.
- [101] Further, another agent capable of increasing bioavailability or maintaining activity may be a formulation such as an implant, an inhalation, a nasal spray, and a patch.
- [102] As used herein, the term "diabetes" means a metabolic disease caused by a lack in the secretion of insulin or abnormality in the function of insulin. Concurrent administration of the composition of the present invention to a subject is performed to control the blood glucose level, thereby treating diabetes.
- [103] As used herein, the term "prevention" refers to all of the actions by which the occurrence of diabetes is restrained or retarded by concurrent administration of the composition of the present invention, and the term "treatment" refers to all of the actions by which the symptoms of diabetes have taken a turn for the better or been modified favorably by concurrent administration of the composition of the present invention. The treatment of diabetes may be applied to any mammal that may have diabetes, and examples thereof include humans and primates as well as livestock such as cattle, pigs, sheep, horses, dogs, and cats without limitation, and preferably humans.
- [104] Further, the pharmaceutical composition including the conjugate of the present invention may include a pharmaceutically acceptable carrier. For oral administration, the pharmaceutically acceptable carrier may include a binder, a lubricant, a disintegrant, an excipient, a solubilizer, a dispersing agent, a stabilizer, a suspending agent, a coloring agent, and a flavor. For injectable preparations, the pharmaceutically acceptable carrier may include a buffering agent, a preserving agent, an analgesic, a solubilizer, an isotonic agent, and a stabilizer. For preparations for topical administration, the pharmaceutically acceptable carrier may include a base, an excipient, a lubricant, and a preserving agent. The pharmaceutical composition of the present

invention may be formulated into a variety of dosage forms in combination with the aforementioned pharmaceutically acceptable carriers. For example, for oral administration, the pharmaceutical composition may be formulated into tablets, troches, capsules, elixirs, suspensions, syrups or wafers. For injectable preparations, the pharmaceutical composition may be formulated into an ampule as a single-dose dosage form or a multi-dose container. The pharmaceutical composition may be also formulated into solutions, suspensions, tablets, pills, capsules, and long-acting preparations.

[105] On the other hand, examples of the carrier, the excipient, and the diluent suitable for formulations include lactose, dextrose, sucrose, sorbitol, mannitol, xylitol, erythritol, maltitol, starch, acacia, alginate, gelatin, calcium phosphate, calcium silicate, cellulose, methylcellulose, microcrystalline cellulose, polyvinylpyrrolidone, water, methylhydroxybenzoate, propylhydroxybenzoate, talc, magnesium stearate, and mineral oils.

[106] In addition, the pharmaceutical formulations may further include fillers, anti-coagulating agents, lubricants, humectants, flavors, and antiseptics.

[107]

[108] In still another aspect, the present invention provides a method for treating insulin-related diseases, including the step of administering the insulin analogue conjugate, the long-acting insulin formulation, or the long-acting formulation for preventing or treating diabetes to a subject in need thereof.

[109] The conjugate according to the present invention is useful in the treatment of diabetes, and therefore, the disease may be treated by administering the pharmaceutical composition including the same.

[110] The term "administration", as used herein, refers to an introduction of a predetermined substance into a patient by a certain suitable method. The conjugate may be administered via any of the common routes, as long as it can reach a desired tissue. Intraperitoneal, intravenous, intramuscular, subcutaneous, intradermal, oral, topical, intranasal, intrapulmonary, and intrarectal administration may be performed, but is not limited thereto. However, since peptides are digested upon oral administration, active ingredients of a composition for oral administration should be coated or formulated for its protection against degradation in the stomach. Preferably, the composition may be administered in an injectable form. In addition, the pharmaceutical composition may be administered using a certain apparatus capable of transporting the active ingredients into a target cell.

[111] Further, the pharmaceutical composition of the present invention may be determined by several related factors including the types of diseases to be treated, administration routes, the patient's age, gender, weight, and severity of the illness, as well as by the types of the drug as an active component. Since the pharmaceutical composition of the

present invention has excellent in-vivo duration and titer, it may greatly reduce administration frequency of the pharmaceutical formulation of the present invention.

[112]

Mode for the Invention

[113] Hereinafter, the present invention will be described in more detail with reference to the following Examples. However, these Examples are for illustrative purposes only, and the invention is not intended to be limited by these Examples.

[114]

[115] **Example 1: Preparation of single-chain insulin analogue expression vector**

[116] In order to prepare insulin analogues, each analogue having a modification of one amino acid in A chain or B chain, using the available native insulin expression vector as a template, forward and reverse oligonucleotides were synthesized (Table 2), and PCR was performed to amplify respective analogue genes.

[117] Each of the amino acid sequences modified in A chain or B chain and the name of each analogue are given in the following Table 1. That is, Analogue 1 has a substitution of alanine for glycine at position 1 of A chain, and Analogue 4 has a substitution of alanine for glycine at position 8 of B chain.

[118] [Table 1]

Analogue	Modified sequence
Analogue 1	A ¹ G → A
Analogue 2	A ² I → A
Analogue 3	A ¹⁹ Y → A
Analogue 4	B ⁸ G → A
Analogue 5	B ²³ G → A
Analogue 6	B ²⁴ F → A
Analogue 7	B ²⁵ F → A
Analogue 8	A ¹⁴ Y → E
Analogue 9	A ¹⁴ Y → N

[119] Primers for insulin analogue amplification are given in the following Table 2.

[120] [Table 2]

Analogue	Sequence	SEQ ID NO
Analogue 1	5' GGGTCCCTGCAGAAGCGTGGGATTGTGGAACAATGCTGT 3'	SEQ ID NO 1
	5' ACAGCATTGTTCCACAATCGCACGCTTCTGCAGGGACCC 3'	SEQ ID NO 2
Analogue 2	5' TCCTGCAGAAGCGTGGCGCGGTGGAACAATGCTGTACC 3'	SEQ ID NO 3
	5' GGTACAGCATTGTTCCACCGCGCCACGCTTCTGCAGGGA 3'	SEQ ID NO 4
Analogue 3	5' CTCTACCAGCTGGAAAACGCGTGTAACTGAGGATCC 3'	SEQ ID NO 5
	5' GGATCCTCAGTTACACGCGTTTTCAGCTGGTAGAG 3'	SEQ ID NO 6
Analogue 4	5' GTTAACCAACACTTGTGTGCGTCACACCTGGTGGAGCT 3'	SEQ ID NO 7
	5' AGCTTCCACCAGGTGTGACGCACACAAGTGTGTTAAC 3'	SEQ ID NO 8
Analogue 5	5' CTAGTGTGCGGGGAACGAGCGTTCTTCTACACACCCAG 3'	SEQ ID NO 9
	5' CTTGGGTGTGTAGAAGAACGCTCGTTCCCCGCACACTAG 3'	SEQ ID NO 10
Analogue 6	5' GTGTGCGGGGAACGAGGCGGTTCTACACACCCAGACC 3'	SEQ ID NO 11
	5' GGTCTTGGGTGTGTAGAAGCGCGCTCGTTCCCCGCACAC 3'	SEQ ID NO 12
Analogue 7	5' TGCGGGGAACGAGGCTTCGCGTACACACCCAGACCCGC 3'	SEQ ID NO 13
	5' GCGGGTCTTGGGTGTGTACGCGAAGCCTCGTTCCCCGCA 3'	SEQ ID NO 14
Analogue 8	5'-CCAGCATCTGCTCCCTCGAACAGCTGGAGAACTACTG-3'	SEQ ID NO 15
	5'-Cagtagttctccagctggttagggagcagatgctgg-3'	SEQ ID NO 16
Analogue 9	5'-CAGCATCTGCTCCCTCAACCAGCTGGAGAACTAC-3'	SEQ ID NO 17
	5'-Gtagttctccagctggttagggagcagatgctg-3'	SEQ ID NO 18

[121] PCR for insulin analogue amplification was performed under conditions of at 95°C for 30 seconds, at 55°C for 30 seconds, and at 68°C for 6 minutes for 18 cycles. In order to express the insulin analogue fragments obtained under the conditions as intracellular inclusion bodies, each of the fragments was inserted into pET22b vector, and the thus-obtained expression vectors were designated as pET22b-insulin analogues 1 to 9, respectively. The respective expression vectors included nucleic acids encoding amino acid sequences of Insulin analogues 1 to 9 under control of T7 promoter, and expressed the insulin analogue proteins as inclusion bodies in host cells, respectively.

[122]

[123] DNA sequences and protein sequences of Insulin analogues 1 to 9 are given in the following Table 3.

[124]

[125] [Table 3]

Analogue	Sequence		SEQ ID NO
Analogue 1	DNA	TTC GTT AAC CAA CAC TTG TGT GGC TCA CAC CTG GTG GAA GCT CTC TAC CTA GTG TGC GGG GAA CGA GGC TTC TTC TAC ACA CCC AAG ACC CGC CGG GAG GCA GAG GAC CTG CAG GTG GGG CAG GTG GAG CTG GGC GGG GGC CCT GGT GCA GGC AGC CTG CAG CCC TTG GCC CTG GAG GGG TCC CTG CAG AAG CGT GCG ATT GTG GAA CAA TGC TGT ACC AGC ATC TGC TCC CTC TAC CAG CTG GAG AAC TAC TGC AAC	19
	Protein	Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Arg Arg Glu Ala Glu Asp Leu Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys Arg Ala Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn	20
Analogue 2	DNA	TTC GTT AAC CAA CAC TTG TGT GGC TCA CAC CTG GTG GAA GCT CTC TAC CTA GTG TGC GGG GAA CGA GGC TTC TTC TAC ACA CCC AAG ACC CGC CGG GAG GCA GAG GAC CTG CAG GTG GGG CAG GTG GAG CTG GGC GGG GGC CCT GGT GCA GGC AGC CTG CAG CCC TTG GCC CTG GAG GGG TCC CTG CAG AAG CGT GGC GCG GTG GAA CAA TGC TGT ACC AGC ATC TGC TCC CTC TAC CAG CTG GAG AAC TAC TGC AAC	21
	Protein	Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Arg Arg Glu Ala Glu Asp Leu Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys Arg Gly Ala Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn	22
Analogue 3	DNA	TTC GTT AAC CAA CAC TTG TGT GGC TCA CAC CTG GTG GAA GCT CTC TAC CTA GTG TGC GGG GAA CGA GGC TTC TTC TAC ACA CCC AAG ACC CGC CGG GAG GCA GAG GAC CTG CAG GTG GGG CAG GTG GAG CTG GGC GGG GGC CCT GGT GCA GGC AGC CTG CAG CCC TTG GCC CTG GAG GGG TCC CTG CAG AAG CGT GGC ATT GTG GAA CAA TGC TGT ACC AGC ATC TGC TCC CTC TAC CAG CTG GAG AAC GCG TGC AAC	23
	Protein	Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Arg Arg Glu Ala Glu Asp Leu Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Ala Cys Asn	24

[126]

Analogue 4	DNA	TTC GTT AAC CAA CAC TTG TGT GCG TCA CAC CTG GTG GAA GCT CTC TAC CTA GTG TGC GGG GAA CGA GGC TTC TTC TAC ACA CCC AAG ACC CGC CGG GAG GCA GAG GAC CTG CAG GTG GGG CAG GTG GAG CTG GGC GGG GGC CCT GGT GCA GGC AGC CTG CAG CCC TTG GCC CTG GAG GGG TCC CTG CAG AAG CGT GGC ATT GTG GAA CAA TGC TGT ACC AGC ATC TGC TCC CTC TAC CAG CTG GAG AAC TAC TGC AAC	25
	Protein	Phe Val Asn Gln His Leu Cys Ala Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Arg Arg Glu Ala Glu Asp Leu Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn	26
Analogue 5	DNA	TTC GTT AAC CAA CAC TTG TGT GGC TCA CAC CTG GTG GAA GCT CTC TAC CTA GTG TGC GGG GAA CGA GCG TTC TTC TAC ACA CCC AAG ACC CGC CGG GAG GCA GAG GAC CTG CAG GTG GGG CAG GTG GAG CTG GGC GGG GGC CCT GGT GCA GGC AGC CTG CAG CCC TTG GCC CTG GAG GGG TCC CTG CAG AAG CGT GGC ATT GTG GAA CAA TGC TGT ACC AGC ATC TGC TCC CTC TAC CAG CTG GAG AAC TAC TGC AAC	27
	Protein	Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Ala Phe Phe Tyr Thr Pro Lys Thr Arg Arg Glu Ala Glu Asp Leu Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn	28
Analogue 6	DNA	TTC GTT AAC CAA CAC TTG TGT GGC TCA CAC CTG GTG GAA GCT CTC TAC CTA GTG TGC GGG GAA CGA GGC GCG TTC TAC ACA CCC AAG ACC CGC CGG GAG GCA GAG GAC CTG CAG GTG GGG CAG GTG GAG CTG GGC GGG GGC CCT GGT GCA GGC AGC CTG CAG CCC TTG GCC CTG GAG GGG TCC CTG CAG AAG CGT GGC ATT GTG GAA CAA TGC TGT ACC AGC ATC TGC TCC CTC TAC CAG CTG GAG AAC TAC TGC AAC	29
	Protein	Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Ala Phe Tyr Thr Pro Lys Thr Arg Arg Glu Ala Glu Asp Leu Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn	30

[127]

Analogue 7	DNA	TTC GTT AAC CAA CAC TTG TGT GGC TCA CAC CTG GTG GAA GCT CTC TAC CTA GTG TGC GGG GAA CGA GGC TTC GCG TAC ACA CCC AAG ACC CGC CGG GAG GCA GAG GAC CTG CAG GTG GGG CAG GTG GAG CTG GGC GGG GGC CCT GGT GCA GGC AGC CTG CAG CCC TTG GCC CTG GAG GGG TCC CTG CAG AAG CGT GGC ATT GTG GAA CAA TGC TGT ACC AGC ATC TGC TCC CTC TAC CAG CTG GAG AAC TAC TGC AAC	31
	Protein	Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Ala Tyr Thr Pro Lys Thr Arg Arg Glu Ala Glu Asp Leu Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn	32
Analogue 8	DNA	TTC GTT AAC CAA CAC TTG TGT GGC TCA CAC CTG GTG GAA GCT CTC TAC CTA GTG TGC GGG GAA CGA GGC TTC TTC TAC ACA CCC AAG ACC CGC CGG GAG GCA GAG GAC CTG CAG GTG GGG CAG GTG GAG CTG GGC GGG GGC CCT GGT GCA GGC AGC CTG CAG CCC TTG GCC CTG GAG GGG TCC CTG CAG AAG CGT GGC ATT GTG GAA CAA TGC TGT ACC AGC ATC TGC TCC CTC GAA CAG CTG GAG AAC TAC TGC AAC TGA	33
	Protein	Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Arg Arg Glu Ala Glu Asp Leu Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Glu Gln Leu Glu Asn Tyr Cys Asn	34
Analogue 9	DNA	TTC GTT AAC CAA CAC TTG TGT GGC TCA CAC CTG GTG GAA GCT CTC TAC CTA GTG TGC GGG GAA CGA GGC TTC TTC TAC ACA CCC AAG ACC CGC CGG GAG GCA GAG GAC CTG CAG GTG GGG CAG GTG GAG CTG GGC GGG GGC CCT GGT GCA GGC AGC CTG CAG CCC TTG GCC CTG GAG GGG TCC CTG CAG AAG CGT GGC ATT GTG GAA CAA TGC TGT ACC AGC ATC TGC TCC CTC AAC CAG CTG GAG AAC TAC TGC AAC TGA	35
	Protein	Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Arg Arg Glu Ala Glu Asp Leu Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Asn Gln Leu Glu Asn Tyr Cys Asn	36

[128] **Example 2: Expression of recombinant insulin analogue fusion peptides**

[129] Recombinant insulin analogues were expressed under control of T7 promoter. E.coli BL21-DE3 (E. coli B F-dcm ompT hsdS(rB-mB-) gal λDE3); Novagen) was transformed with each of the recombinant insulin analogue expression vectors. Trans-

formation was performed in accordance with the procedures recommended by Novagen. Single colonies transformed with the respective recombinant expression vectors were inoculated in ampicillin (50 µg/ml)-containing 2X Luria Broth (LB) medium, and cultured at 37°C for 15 hours. Each culture broth of the recombinant strain and 30% glycerol-containing 2X LB medium were mixed at a ratio of 1:1(v/v), and each 1 ml thereof was aliquoted into a cryotube, and stored at -140°C. These samples were used as cell stocks for production of the recombinant fusion proteins.

- [130] To express recombinant insulin analogues, each 1 vial of the cell stocks was thawed and inoculated in 500 ml of 2X Luria Broth, and cultured under shaking at 37°C for 14~16 hours. When OD600 value reached 5.0 or higher, the culture was terminated and the culture broth was used as a seed culture. The seed culture was inoculated to a 50 L fermentor (MSJ-U2, B.E.MARUBISHI, Japan) containing 17 L of fermentation medium to begin initial bath fermentation. The culture conditions were maintained at 37°C, an air flow rate of 20 L/min (1 vvm), and an agitation speed of 500 rpm with a pH adjusted to 6.70 with 30% aqueous ammonia. Fermentation was performed in a fed-batch mode by further adding a feeding solution when nutrients in the culture broth were depleted. The cell growth was monitored by OD measurement. At an OD600 value above 100 or higher, IPTG was introduced at a final concentration of 500 µM. After introduction, culture was further performed for about 23~25 hours. After terminating the culture, recombinant strains were harvested using a centrifuge, and stored at -80°C until use.

[131]

[132] **Example 3: Recovery and refolding of recombinant insulin analogues**

- [133] In order to change the recombinant insulin analogues expressed in Example 2 into soluble forms, cells were disrupted, followed by refolding. 100 g (wet weight) of the cell pellet was resuspended in 1 L of lysis buffer (50 mM Tris-HCl (pH 9.0), 1 mM EDTA (pH 8.0), 0.2 M NaCl and 0.5% Triton X-100). The cells were disrupted using a microfluidizer processor M-110EH (AC Technology Corp. Model M1475C) at an operating pressure of 15,000 psi. The cell lysate thus disrupted was centrifuged at 7,000 rpm and 4°C for 20 minutes. The supernatant was discarded and the pellet was resuspended in 3 L of washing buffer (0.5% Triton X-100 and 50 mM Tris-HCl (pH 8.0), 0.2 M NaCl, 1 mM EDTA). After centrifugation at 7,000 rpm and 4°C for 20 minutes, the cell pellet was resuspended in distilled water, followed by centrifugation in the same manner. The pellet thus obtained was resuspended in 400 ml of buffer (1 M Glycine, 3.78 g Cysteine-HCl, pH 10.6) and stirred at room temperature for 1 hour. To recover the recombinant insulin analogue thus re-suspended, 400 mL of 8 M urea was added and stirred at 40°C for 1 hour. For refolding of the solubilized recombinant insulin analogues, centrifugation was carried out at 7,000 rpm and 4°C for 30 minutes,

and the supernatant was obtained. 7.2 L of distilled water was added thereto using a peristaltic pump at a flow rate of 1000 ml/hr while stirring at 4°C for 16 hours.

[134]

[135] **Example 4: Cation binding chromatography purification**

[136] The sample refolded was loaded onto a Source S (GE healthcare) column equilibrated with 20 mM sodium citrate (pH 2.0) buffer containing 45% ethanol, and then the insulin analogue proteins were eluted in 10 column volumes with a linear gradient from 0% to 100% 20 mM sodium citrate (pH 2.0) buffer containing 0.5 M potassium chloride and 45% ethanol.

[137]

[138] **Example 5: Trypsin and carboxypeptidase B treatment**

[139] Salts were removed from the eluted samples using a desalting column, and exchanged with a buffer (10 mM Tris-HCl, pH 8.0). With respect to the obtained sample protein, trypsin corresponding to 1000 molar ratio and carboxypeptidase B corresponding to 2000 molar ratio were added, and then stirred at 16°C for 16 hours. To terminate the reaction, 1 M sodium citrate (pH 2.0) was used to lower pH to 3.5.

[140]

[141] **Example 6: Cation binding chromatography purification**

[142] The sample thus reacted was loaded onto a Source S (GE healthcare) column equilibrated with 20 mM sodium citrate (pH 2.0) buffer containing 45% ethanol, and then the insulin analogue proteins were eluted in 10 column volumes with a linear gradient from 0% to 100% 20 mM sodium citrate (pH 2.0) buffer containing 0.5 M potassium chloride and 45% ethanol.

[143]

[144] **Example 7: Anion binding chromatography purification**

[145] Salts were removed from the eluted sample using a desalting column, and exchanged with a buffer (10 mM Tris-HCl, pH 7.5). In order to isolate a pure insulin analogue from the sample obtained in Example 6, the sample was loaded onto an anion exchange column (Source Q: GE healthcare) equilibrated with 10 mM Tris (pH 7.5) buffer, and the insulin analogue protein was eluted in 10 column volumes with a linear gradient from 0% to 100% 10 mM Tris (pH 7.5) buffer containing 0.5 M sodium chloride.

[146] Purity of the insulin analogue thus purified was analyzed by protein electrophoresis (SDS-PAGE) and high pressure chromatography (HPLC), and modifications of amino acids were identified by peptide mapping and molecular weight analysis of each peak.

[147]

[148] As a result, each insulin analogue was found to have the desired modification in the amino acid sequence.

[149]

[150] **Example 8: Comparison of insulin receptor binding affinity between native insulin and insulin analogues**

[151] In order to measure the insulin receptor binding affinity of the representative insulin analogues of the present invention, Surface plasmon resonance (SPR, BIACORE 3000, GE healthcare) was used for analysis. Insulin receptors were immobilized on a CM5 chip by amine coupling, and 5 dilutions or more of native insulin and insulin analogues were applied thereto, independently. Then, the insulin receptor binding affinity of each substance was examined. The binding affinity of each substance was calculated using BIAevaluation software. In this regard, the model used was 1:1 Langmuir binding with baseline drift.

[152] As a result, compared to human insulin, Insulin analogue (No. 6), insulin analogue (No. 7), Insulin analogue (No. 8) and Insulin analogue (No. 9) showed receptor binding affinity of 14.8%, 9.9%, 57.1%, and 78.8%, respectively (Table 4). As such, it was confirmed that the insulin analogues of the present invention have remarkably reduced insulin receptor binding affinity, compared to the native insulin.

[153] [Table 4]

Test No.	Name	k_a (1/Ms, $\times 10^5$)	k_d (1/s, $\times 10^{-3}$)	K_D (nM)
Test 1	Native human insulin	2.21 (100%)	7.47 (100%)	35.05 (100%)
	Insulin analogue (No. 6)	0.28 (12.6%)	6.60 (88.4%)	237.0 (14.8%)
Test 2	Native human insulin	1.76 (100%)	10.73 (100%)	63.47 (100%)
	Insulin analogue (No. 7)	0.14 (7.8%)	8.34 (77.7%)	642.0 (9.9%)
Test 3	Native human insulin	2.9 (100%)	12.4 (100%)	42.0 (100%)

[154]

Test 4	Insulin analogue (No. 8)	1.78 (60.0%)	12.9 (104.6%)	73.4 (57.1%)
	Native human insulin	2.0 (100%)	9.7 (100%)	50.4 (100%)
	Insulin analogue (No. 9)	1.85 (92.5%)	11.9 (122.5%)	64.0 (78.8%)

[155]

[156] The above results indicate that the insulin analogues of the present invention have reduced in-vivo insulin receptor binding affinity, compared to native insulin, and thus they avoid RMC, thereby increasing in-vivo half-life.

[157]

[158] **Example 9: Preparation of representative insulin analogue(No. 7)-PEG conjugate**

[159] To pegylate the lysine residue of B chain of the insulin analogue using mPEG-SBA (20kDa, Nektar, USA), the insulin analogue and PEG were reacted at a molar ratio of 1:2 with an insulin analogue concentration of 1.5 mg/ml at room temperature for about 1 hour. In this regard, the reaction was performed in 50 mM sodium borate at pH 9.0. 20.0 mM sodium cyanoborohydride was added as a reducing agent and was allowed to react. The reaction solution was purified with Source15S (GE Healthcare, USA) column using a buffer containing sodium citrate (pH 3.0) and 45% ethanol, and KCl concentration gradient.

[160] Protein electrophoresis (SDS-PAGE) and high pressure chromatography (HPLC) were performed to confirm that the insulin analogue-PEG conjugate purified from Source15S column had a purity > 95%.

[161]

[162] **Example 10: Preparation of representative insulin analogue(No. 7)-ELP conjugate**

[163] To prepare an elastin-binding protein, an ELP motif expression vector was prepared. To prepare an ELP motif consisting of 10 amino acids, an oligonucleotide (SEQ ID NO: 37) was prepared and used as a template to prepare forward and reverse primers (SEQ ID NOS: 38 and 39).

[164] PCR for ELP motif gene amplification was performed under conditions of at 95°C for 15 seconds, at 54°C for 30 seconds, and at 68°C for 15 seconds for 25 cycles. The oligonucleotide and primers used in PCR are given in the following Table 5.

[165] [Table 5]

	Sequence	SEQ ID NO
Oligonucleotide	5'-cagcatctgatctgcccggatcccggggttgagtgccctggtgtagggcgtcccag gcccagggtgtgccgggagcagggtgtccagggtgtaggtgtccctgggggttgagtgcc ctggtgtagggcgtcccaggcggagggtgtgccgggagcagggtgtccagggtggaggcg tgcccgggatcccgtagcatctggtcgc-3'	37
Forward Primer	5'-CAGCATCTGATCTGcccgat-3'	38
Reverse Primer	5'-GCGACCAGATGCTACGGGAT-3'	39

[166] A PCR product was extracted using a gel extraction kit (Qiagen, Germany), and then treated with the restriction enzyme BamHI to prepare an insert fragment. Next, pET22b vector (Novagen, USA) was digested with the same enzyme and then extracted using the same gel extraction kit. The insert fragment and vector were ligated using T4 ligase, and transformed into E.coli TOP10 competent cells (Invitrogen, USA). The transformant was inoculated on an LB-ampicillin plate, followed by incubation. Colonies thus formed were randomly selected and incubated in an LB-ampicillin medium overnight. Plasmid DNA was extracted therefrom. The plasmid DNA was digested with the restriction enzyme BamHI, and electrophoresis was performed to confirm whether insert DNA had a desired size. Then, insertion of ELP motif into the vector was confirmed by DNA sequencing. The present inventors designated this vector including the ELP motif thus prepared as 'pET22b-ELP motif'.

[167] To prepare an ELP-binding protein having 12 repeats of the ELP motif (50 amino acids), the ELP motif-inserted vector was digested with the restriction enzyme XmaI to extract the ELP motif. The extracted ELP motif was mixed with double strand oligonucleotides (SEQ ID NOS: 40~43, Table 6) having BamHI and XmaI restriction sites, and then ligated using T4 ligase to prepare insert fragments having different sizes. As annealing conditions for preparation of double strands, oligonucleotides were mixed

with 10 mM Tris pH 8.0, 50 mM NaCl and 1 mM EDTA, and then left at 95°C for 5 minutes. Then, the temperature was slowly decreased to 4°C. Each of the prepared insert fragments having different sizes was ligated with pET22b vector that had been digested with the restriction enzyme BamHI, and transformed into E.coli TOP10 competent cells. Each transformant was inoculated on an LB-ampicillin plate, followed by incubation. Colonies thus formed were randomly selected and incubated in an LB-ampicillin medium overnight. Plasmid DNAs were extracted therefrom. The plasmid DNAs were digested with the restriction enzyme BamHI, and electrophoresis was performed to confirm whether insert DNA had a desired size. Then, insertion of ELP-binding protein into the vector was confirmed by DNA sequencing. The present inventors designated this vector including the ELP-binding protein thus prepared as 'ET22b-ELP-BP'.

[168] [Table 6]

Name	Sequence	SEQ ID NO
Double stranded oligonucleotide	5' – GATCCATCCATGCTACGTTC – 3'	40
Double stranded oligonucleotide	5' – CCGGGAACGTAGCATGGATG – 3'	41
Double stranded oligonucleotide	5' – GATCCGACATGTCACGGCCAC – 3'	42
Double stranded oligonucleotide	5' –GATCCGACATGTCACGGCCAC– 3'	43

[169]

[170] **Example 11: Preparation of insulin analogue-ELP-BP fusion protein expression vector**

[171] To prepare a fusion protein formed by linking ELP-BP to the C-terminus of the insulin analogue, PCR was performed using an insulin N-terminus primer (SEQ ID NO: 44) containing the NdeI restriction enzyme site and an insulin C-terminus primer (SEQ ID NO: 45) containing the N-terminal sequence of ELP-BP so as to amplify insulin, and PCR was also performed using an ELP-BP N-terminus primer (SEQ ID NO: 46) containing insulin C-terminal region and an ELP-BP C-terminus primer (SEQ ID NO: 47) containing the BamHI restriction enzyme site so as to amplify ELP-BP gene (Table 7). PCR was performed under conditions of at 95°C for 30 seconds, at 55°C for 30 seconds, and at 68°C for 30 seconds and 6 minutes for 30 cycles. Secondary PCR was performed using the amplified gene as a template so as to amplify

an insulin-ELP-BP fusion gene. Secondary PCR was performed under conditions of at 95°C for 30 seconds, at 55°C for 30 seconds, and at 68°C for 2 minutes and 30 seconds for 30 cycles.

[172] The amplified gene was digested with NdeI and BamHI, and then ligated with pET22B vector, which had been digested with the same restriction enzymes, using T4 ligase, followed by transformation into E.coli TOP10 competent cells (Invitrogen, USA). In the same manner as in Example 10, the sequence of the fusion protein was examined, and then the fusion protein was designated as 'pET22b-Insulin-ELPBP'. DNA and protein sequences thereof are represented by SEQ ID NOS: 59 and 60, respectively.

[173] [Table 7]

Name	Sequence	SEQ ID NO
Insulin N-terminal primer	5' – GAGATATACATATGATGGCAACAACATCAAC – 3'	44
Insulin C-terminal primer	5' – CCCCAGGAACGTTGCAGTAGTTCTCCAG – 3'	45
ELP-BP N-terminal primer	5' – GAGAACTACTGCAACggtcccggggtagtg – 3'	46
ELP-BP C-terminal primer	5' –CGCGGATCCGACATGTCACGGCCAC– 3'	47

[174] The above results suggest that the insulin analogue conjugate of the present invention has a reduced insulin receptor binding affinity, and thus it avoids in-vivo RMC and renal clearance to remarkably increase blood half-life, thereby providing a long-acting formulation including the insulin analogue conjugate.

[175]

[176] Based on the above description, it will be understood by those skilled in the art that the present invention may be implemented in a different specific form without changing the technical spirit or essential characteristics thereof. Therefore, it should be understood that the above embodiment is not limitative, but illustrative in all aspects. The scope of the invention is defined by the appended claims rather than by the description preceding them, and therefore all changes and modifications that fall within metes and bounds of the claims, or equivalents of such metes and bounds are therefore intended to be embraced by the claims.

Claims

- [Claim 1] An insulin analogue conjugate having a reduced insulin receptor binding affinity, compared to a native insulin, wherein the conjugate has the following Chemical Formula:
[Chemical Formula]
X-La-F
wherein X is an insulin analogue having a reduced insulin receptor binding affinity, compared to the native insulin;
L is a linker, and a is 0 or a natural number (if a is 2 or larger, each L is independent); and
F is polyethylene glycol, an amino acid polymer, or a combination thereof.
- [Claim 2] The conjugate of claim 1, wherein the insulin analogue has an alteration selected from the group consisting of substitution, addition, deletion, modification and combinations thereof in one or more amino acids of the native insulin.
- [Claim 3] The conjugate of claim 1, wherein the insulin analogue has a mutation or deletion of one or more amino acids.
- [Claim 4] The conjugate according to claim 1, wherein the insulin analogue is prepared by substitution of one or more amino acids selected from the group consisting of amino acids at positions 1, 2, 3, 5, 8, 10, 12, 16, 23, 24, 25, 26, 27, 28, 29, and 30 of B chain and at positions 1, 2, 5, 8, 10, 12, 14, 16, 17, 18, 19 and 21 of A chain with other amino acid(s), or by deletion thereof.
- [Claim 5] The conjugate of claim 4, wherein the substitution to other amino acid(s) is substitution to amino acid(s) selected from the group consisting of alanine, glutamic acid, asparagine, isoleucine, valine, glutamine, glycine, lysine, histidine, cysteine, phenylalanine, tryptophan, proline, serine, threonine, and aspartic acid.
- [Claim 6] The conjugate of claim 1, wherein the F is selected from the group consisting of polyethylene glycol, elastin, a water-soluble precursor of elastin, and a polymer of repeating units of a part of the elastin protein sequence.
- [Claim 7] The conjugate of claim 6, wherein the repeating unit of a part of the elastin protein sequence is VPGXG of SEQ ID NO: 49 (wherein X is alanine, arginine, asparagine, aspartic acid, glutamic acid, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, or

- valine).
- [Claim 8] The conjugate of claim 7, wherein the repeating unit is selected from the group consisting of VPGVG (SEQ ID NO: 50), VPGAG (SEQ ID NO: 51), VPGGG (SEQ ID NO: 52), VPGIG (SEQ ID NO: 53), VPGLG (SEQ ID NO: 54), VPGFG (SEQ ID NO: 55), AVGVP (SEQ ID NO: 56), IPGVG (SEQ ID NO: 57), LPGVG (SEQ ID NO: 58), and combinations thereof.
- [Claim 9] The conjugate of claim 1, wherein the conjugate has a long-acting property of increased in-vivo half-life.
- [Claim 10] The conjugate of claim 1, wherein the linker is a non-peptidyl polymer.
- [Claim 11] The conjugate of claim 10, wherein the non-peptidyl polymer is selected from the group consisting of polyethylene glycol, fatty acids, saccharides, polymers, low-molecular-weight compounds, nucleotides and combinations thereof.
- [Claim 12] The conjugate of claim 11, wherein the polymer is selected from the group consisting of polypropylene glycol, an ethylene glycol-propylene glycol copolymer, polyoxyethylated polyol, polyvinyl alcohol, polysaccharide, dextran, polyvinyl ethyl ether, a biodegradable polymer, a lipid polymer, chitin, hyaluronic acid, an oligonucleotide and combinations thereof.
- [Claim 13] The conjugate of claim 1, wherein polyethylene glycol has a molecular weight of 10 to 100,000 Da.
- [Claim 14] The conjugate of claim 13, wherein polyethylene glycol is linked to one or more amino acid residues selected from the group consisting of the N-terminal amino acid of the insulin analogue and the C-terminal amino acid of the insulin analogue; and lysine, cysteine, aspartic acid, and glutamic acid within the insulin analogue.
- [Claim 15] The conjugate of claim 1, wherein X is linked to F via L by a covalent bond, a non-covalent bond or a combination thereof.
- [Claim 16] A long-acting insulin formulation having increased in-vivo duration and stability, comprising the insulin analogue conjugate of any one of claims 1 to 15.
- [Claim 17] A long-acting formulation for preventing or treating diabetes, comprising the insulin analogue conjugate of any one of claims 1 to 15.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2015/006619**A. CLASSIFICATION OF SUBJECT MATTER****C07K 14/62(2006.01)i, A61K 38/28(2006.01)i, A61P 3/10(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K 14/62; A61K 38/28; A61K 31/25; A61P 3/10

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: long-acting insulin analogue, reduced insulin receptor binding affinity, PEG, linker

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2012-0184488 A1 (WEISS) 19 July 2012 See claims 16-29; and paragraphs [0032]-[0047].	1-17
Y	WO 2008-084051 A1 (NOVO NORDISK A/S) 17 July 2008 See pages 2 and 7-10.	1-17
A	VAREWIJCK et al., 'Insulin and its analogues and their affinities for the IGF1 receptor' Endocrine-Related Cancer, Vol.19, No.5, pp.F63-F75 (2012) See the whole document.	1-17
A	KR 10-2005-0121748 A (ELI LILLY AND COMPANY) 27 December 2005 See the whole document.	1-17
A	JP 2012-062311 A (CONJUCHEM LLC) 29 March 2012 See the whole document.	1-17



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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"&" document member of the same patent family

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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