(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 January 2016 (14.01.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/006963 A1

(51) International Patent Classification:
*C07K 14/62* (2006.01) *C07K 19/00* (2006.01)
*C12N 15/09* (2006.01) *A61K 38/28* (2006.01)

(21) International Application Number:
PCT/KR2015/007182

(22) International Filing Date:
10 July 2015 (10.07.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10-2014-0087793 11 July 2014 (11.07.2014) KR

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,

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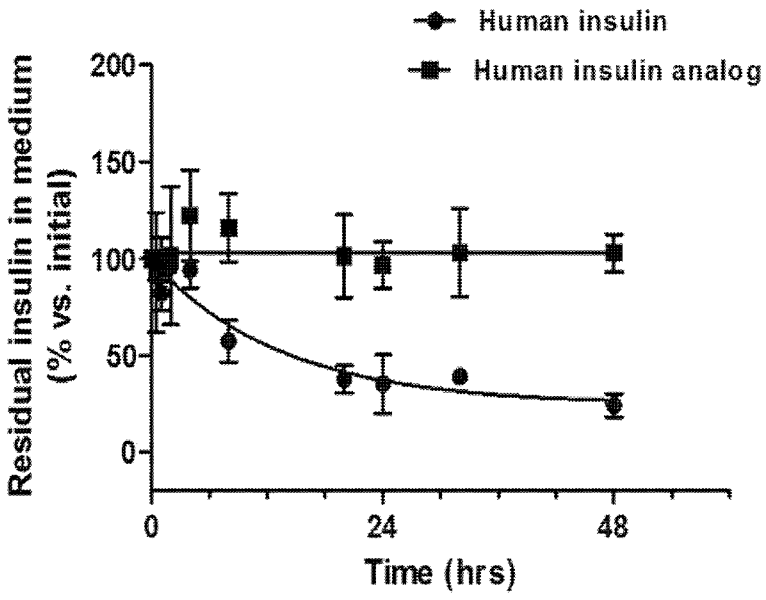
(54) Title: INSULIN ANALOGUE




(57) Abstract: The present invention relates to an insulin analogue having a reduced insulin receptor-mediated clearance rate, compared to native insulin, and an insulin analogue conjugate thereof. Further, the present invention relates to a long-acting insulin analogue formulation using the insulin analogue or the conjugate thereof, a long-acting formulation for preventing or treating diabetes including the conjugate, and a long-acting formulation for preventing or treating diabetes including the insulin analogue or the conjugate thereof; and an insulinotropic peptide or a derivative thereof. Furthermore, the present invention relates to a method for treating diabetes using the insulin analogue, the conjugate thereof, the long-acting insulin formulation, or the long-acting formulation for preventing or treating diabetes. The insulin analogue of the present invention has a reduced insulin receptor-mediated clearance rate to have a increased blood half-life, compared to native insulin, thereby providing an insulin analogue conjugate and a long-acting formulation capable of increasing in-vivo half-life and bioavailability, and/or maintaining activity. Accordingly, dosing convenience for patients with various insulin-related diseases is increased to remarkably improve drug compliance.

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LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

**Published**:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

# Description

## Title of Invention: INSULIN ANALOGUE

### Technical Field

[1] The present invention relates to an insulin analogue having a reduced insulin receptor-mediated clearance rate, compared to native insulin, and an insulin analogue conjugate thereof. Further, the present invention relates to a long-acting insulin analogue formulation using the insulin analogue or the conjugate thereof, a long-acting formulation for preventing or treating diabetes including the conjugate, and a long-acting formulation for preventing or treating diabetes including the insulin analogue or the conjugate thereof; and an insulinotropic peptide or a derivative thereof. Furthermore, the present invention relates to a method for treating diabetes using the insulin analogue, the conjugate thereof, 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 (fatty acid conjugate, 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-lives, the disadvantage of one or two injections per day still remains. Accordingly, there is a limitation in achieving the intended goal that administration frequency is remarkably reduced to improve convenience of diabetic patients in need of long-term administration.

[5]

[6] On the other hand, an in-vivo insulin clearance process is reported in Biochem J (1998) 332, 421, Endocrine Reviews (1998) 19, 608, and 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.

[7] In this regard, many studies, including J Pharmacol Exp Ther (1998) 286: 959, Diabetes Care (1990) 13: 923, and 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.

[8] 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.

[9]

[10] It is known that insulin works to remove glucose in the blood by binding to insulin receptors, and its effectiveness can be controlled by changing the sequence of the native insulin. That is, intrinsic properties of insulin can be controlled by substitution of amino acids of insulin with other amino acids or by deletion of a particular amino acid, and an insulin analogue having a reduced insulin receptor binding affinity and a reduced receptor-mediated clearance is used to maintain in-vivo activity of insulin, and thus it is a therapeutically useful form.

[11]

## Disclosure of Invention

## Technical Problem

[12] The present inventors have made many efforts to increase the blood half-life of insulin. As a result, they prepared a novel insulin analogue having a reduced insulin receptor binding affinity and reduced receptor-mediated clearance (RMC) of insulin, and they found that the insulin analogue has effects of increasing the blood half-life and bioavailability or maintaining the activity, thereby completing the present invention.

[13]

## Solution to Problem

[14] An object of the present invention is to provide an insulin analogue having a reduced insulin receptor-mediated clearance rate, compared to native insulin.

[15] Another object of the present invention is to provide an insulin analogue conjugate formed by linking the insulin analogue having a reduced insulin receptor-mediated clearance rate, compared to native insulin, and polyethylene glycol or an amino acid polymer or a combination thereof.

[16] Still another object of the present invention is to provide a long-acting insulin formulation including the insulin analogue or the conjugate thereof, in which the formulation has increased in-vivo duration and stability.

[17] Still another object of the present invention is to provide a long-acting formulation for preventing or treating diabetes, including the insulin analogue or the conjugate thereof.

[18] Still another object of the present invention is to provide a long-acting formulation for preventing or treating diabetes, including the insulin analogue or the conjugate thereof; and an insulinotropic peptide or a derivative thereof.

[19] 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, the conjugate thereof, 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

[20] An insulin analogue of the present invention has a reduced insulin receptor-mediated clearance rate to have a increased blood half-life, compared to native insulin, thereby providing an insulin analogue conjugate and a long-acting formulation capable of increasing in-vivo half-life and bioavailability, and/or maintaining activity. Accordingly, dosing convenience for patients with various insulin-related diseases is increased to remarkably improve drug compliance.

[21]

## Brief Description of Drawings

[22] FIG. 1 is a graph showing the result of measuring residual concentrations of native insulin and an insulin analogue of the present invention (represented by Insulin analogue 8, SEQ ID NO: 34) over time, in which the concentrations measured over time are expressed as a percentage (%) when a final concentration is taken as 100% (■: native insulin, ●: insulin analogue (Insulin analogue 8, SEQ ID NO: 34)); and

[23] FIG. 2 shows the result of pharmacokinetic analysis of native insulin and an insulin analogue in a nephrectomized animal model (SD rat, male, 6-week-old) (□: native insulin (172 nmol/kg) in a Sham group, ○: insulin analogue (172 nmol/kg) in a Sham

group. ■: native insulin (172 nmol/kg) in a nephrectomized group, ●: insulin analogue (172 nmol/kg) in a nephrectomized group).

[24]

## Best Mode for Carrying out the Invention

[25] In order to achieve the above objects, an aspect of the present invention provides an insulin analogue having a reduced insulin receptor-mediated clearance rate, compared to native insulin. Such insulin analogue has a non-native insulin sequence and a reduced insulin receptor-mediated insulin clearance rate, compared to native insulin, thereby inducing an increase in blood half-life and increasing therapeutic efficacy of insulin. The insulin analogue may be an insulin analogue which has modified amino acids in B chain or A chain of native insulin so as to have a reduced insulin receptor-mediated clearance rate, compared to native insulin.

[26]

[27] As used herein, the term "insulin" means a peptide that is secreted by the pancreas in response to elevated glucose levels in the blood to take up glucose in the liver, muscle, or adipose tissue and turn it into glycogen, and to suppress the use of fat as an energy source, and thus controls the blood glucose level. In the present invention, insulin may be native insulin or an insulin analogue prepared by mutating one or more amino acids in the native insulin.

[28] As used herein, the term "insulin analogue" is a substance that is obtained by mutating one or more amino acids in the native sequence.

[29] The mutation of one or more amino acids in the native sequence means that an alternation selected from the group consisting of substitution, addition, deletion, modification and combinations thereof occurs in one or more amino acids of native insulin.

[30] 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.

[31] The amino acid sequences of the native insulin are as follows.

[32]

[33] A chain:

[34] Gly-Ile-Val-Glu-Gln-Cys-Cys-Thr-Ser-Ile-Cys-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn (SEQ ID NO: 46)

[35] B chain:

[36] 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: 47)

[37]

[38] 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.

[39] 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.

[40] The insulin agonist of the present invention means a compound that binds to the insulin receptor to show the biological activity equal to that of insulin, which is ir-relevant to the structure of insulin.

[41] The insulin analogue of the present invention means a peptide having homology with respective amino acid sequences of the A chain and the 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.

[42] In the present invention, the insulin analogue means 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.

[43] The insulin fragment of the present invention means 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., D-type amino acid), and this insulin fragment has a function of regulating the blood glucose level in the body.

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

[45] 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.

[46]

[47] Specifically, the insulin analogue may be prepared by substitution of one or more amino acid(s) 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 the B chain and at positions 1, 2, 5, 8, 10, 12, 14, 16, 17, 18, 19 and 21 of the A chain with other amino acids. 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 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 also be included in the scope of the present invention, but there is no limitation in the insulin analogue having reduced insulin receptor binding affinity.

[48] The insulin analogue may have a substitution of glutamic acid for tyrosine as an amino acid residue at position 14 of the A chain of native insulin, and may also have a mutation selected from the group consisting of substitution, addition, deletion, modification and combinations thereof in one or more amino acids, in addition to the substitution of glutamic acid for tyrosine as an amino acid residue at position 14 of A chain of native insulin. Further, the insulin analogue of the present invention may be a single-chain or a double-chain.

[49] The insulin analogue may be a single-chain analogue having an amino acid sequence of SEQ ID NO: 34 or a double-chain insulin analogue including A chain consisting of an amino acid sequence of SEQ ID NO: 44 and B chain consisting of an amino acid sequence of SEQ ID NO: 47. In particular, the insulin analogue may be Insulin analogue 8 prepared in the present invention (Table 1).

[50]

[51] In a specific embodiment of the present invention, an experiment of measuring receptor-mediated clearance (RMC) of native insulin and an insulin analogue of the present invention (Insulin analogue 8, single-chain: SEQ ID NO: 34, double-chain: A chain consisting of amino acid sequence of SEQ ID NO: 44 and B chain consisting of amino acid sequence of SEQ ID NO: 47) was performed. As a result, a residual amount of native insulin in a medium was reduced over time, whereas the concentration of the insulin analogue was maintained at an initial concentration until 48 hours (FIG. 1). It was found that the concentration change (reduction) of the insulin analogue of the present invention is less affected by receptor-mediated clearance (RMC), compared to native insulin, which is attributed to the substitution of glutamic acid for tyrosine at position 14 of A chain of insulin.

[52]

[53] Further, in a specific embodiment of the present invention, insulin receptor binding affinities of native insulin and an insulin analogue of the present invention were measured. As a result, when the receptor binding affinity of native insulin was taken as

100%, the receptor binding affinity of Insulin analogue (No. 8) was 57.1% (Table 5). As such, the insulin analogue of the present invention was found to have a remarkably reduced insulin receptor binding affinity, compared to native insulin.

[54]

[55] Furthermore, in a specific embodiment of the present invention, pharmacokinetics of native insulin and an insulin analogue in nephrectomized rats (SD rat, male, 6-week-old) was examined. As a result, in both a sham-operated group and a nephrectomized group, serum concentrations of Insulin analogue 8 were higher than those of native insulin at each time point. The maximum serum concentration and AUC (area under curve) of Insulin analogue 8 were also higher than those of native insulin (FIG. 2), suggesting that its concentration change (reduction) is less affected by receptor-mediated clearance, compared to native insulin.

[56]

[57] Specifically, in an embodiment of the present invention, the insulin analogue may be a single-chain insulin analogue having an amino acid sequence selected from the group consisting of SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 and SEQ ID NO: 36.

[58] Further, in an embodiment of the present invention, the insulin analogue may be a double-chain insulin analogue. For example, the insulin analogue may be selected from the group consisting of an insulin analogue including A chain consisting of an amino acid sequence of SEQ ID NO: 37 and B chain consisting of an amino acid sequence of SEQ ID NO: 47; an insulin analogue including A chain consisting of an amino acid sequence of SEQ ID NO: 38 and B chain consisting of an amino acid sequence of SEQ ID NO: 47; an insulin analogue including A chain consisting of an amino acid sequence of SEQ ID NO: 39 and B chain consisting of an amino acid sequence of SEQ ID NO: 47; an insulin analogue including A chain consisting of an amino acid sequence of SEQ ID NO: 46 and B chain consisting of an amino acid sequence of SEQ ID NO: 40; an insulin analogue including A chain consisting of an amino acid sequence of SEQ ID NO: 46 and B chain consisting of an amino acid sequence of SEQ ID NO: 41; an insulin analogue including A chain consisting of an amino acid sequence of SEQ ID NO: 46 and B chain consisting of an amino acid sequence of SEQ ID NO: 42; an insulin analogue including A chain consisting of an amino acid sequence of SEQ ID NO: 46 and B chain consisting of an amino acid sequence of SEQ ID NO: 43; an insulin analogue including A chain consisting of an amino acid sequence of SEQ ID NO: 44 and B chain consisting of an amino acid sequence of SEQ ID NO: 47; and an insulin analogue including A chain consisting of an amino acid sequence of SEQ ID NO: 45 and B chain consisting of an amino acid sequence of SEQ ID NO: 47.

[59]

[60] In a specific embodiment of the present invention, insulin analogues, each having an amino acid mutated in A chain or B chain, were prepared using a native insulin expression vector as a template. 9 types of analogues were prepared using a single-chain insulin analogue expression vector (Table 1). Analogue 1 having an amino acid sequence of SEQ ID NO: 20, Analogue 2 having an amino acid sequence of SEQ ID NO: 22, Analogue 3 having an amino acid sequence of SEQ ID NO: 24, Analogue 4 having an amino acid sequence of SEQ ID NO: 26, Analogue 5 having an amino acid sequence of SEQ ID NO: 28, Analogue 6 having an amino acid sequence of SEQ ID NO: 30, Analogue 7 having an amino acid sequence of SEQ ID NO: 32, Analogue 8 having an amino acid sequence of SEQ ID NO: 34, and Analogue 9 having an amino acid sequence of SEQ ID NO: 36 were prepared.

[61] Further, in a specific embodiment of the present invention, as the double-chain insulin analogue of the present invention, Insulin analogue 1 including A chain consisting of an amino acid sequence of SEQ ID NO: 37 and B chain consisting of an amino acid sequence of SEQ ID NO: 47; Insulin analogue 2 including A chain consisting of an amino acid sequence of SEQ ID NO: 38 and B chain consisting of an amino acid sequence of SEQ ID NO: 47; Insulin analogue 3 including A chain consisting of an amino acid sequence of SEQ ID NO: 39 and B chain consisting of an amino acid sequence of SEQ ID NO: 47; Insulin analogue 4 including A chain consisting of an amino acid sequence of SEQ ID NO: 46 and B chain consisting of an amino acid sequence of SEQ ID NO: 40; Insulin analogue 5 including A chain consisting of an amino acid sequence of SEQ ID NO: 46 and B chain consisting of an amino acid sequence of SEQ ID NO: 41; Insulin analogue 6 including A chain consisting of an amino acid sequence of SEQ ID NO: 46 and B chain consisting of an amino acid sequence of SEQ ID NO: 42; Insulin analogue 7 including A chain consisting of an amino acid sequence of SEQ ID NO: 46 and B chain consisting of an amino acid sequence of SEQ ID NO: 43; Insulin analogue 8 including A chain consisting of an amino acid sequence of SEQ ID NO: 44 and B chain consisting of an amino acid sequence of SEQ ID NO: 47; and Insulin analogue 9 including A chain consisting of an amino acid sequence of SEQ ID NO: 45 and B chain consisting of an amino acid sequence of SEQ ID NO: 47 were prepared.

[62]

[63] In another aspect, the present invention provides an insulin analogue conjugate having a reduced insulin receptor-mediated clearance rate, compared to the native insulin.

[64] Specifically, the insulin analogue conjugate may be an insulin analogue conjugate having a reduced insulin receptor-mediated clearance rate, compared to the native

insulin, in which the conjugate has the following Chemical Formula:

[65] [Chemical Formula]

[66] X-La-F;

[67] wherein X is selected from the group consisting of the insulin analogue according to the present invention, and derivatives, fragments, and variants thereof,

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

[69] F is a biocompatible material or a carrier (in particular, polyethylene glycol, an amino acid polymer, or a combination thereof).

[70]

[71] In the present invention, the insulin analogue, and derivatives, fragments and variants thereof are the same as described above.

[72] 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.

[73]

[74] The insulin analogue conjugate of the present invention is found to have a new structure, in which receptor-mediated clearance (RMC) is avoided due to the reduced insulin receptor binding affinity, 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 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.

[75]

[76] 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. Examples thereof may include polyethylene glycol or an amino acid polymer, or a combination thereof, but are not limited thereto.

[77] As used herein, the term "biocompatible material or carrier" denotes a substance that is able to increase 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 purpose is to increase half-life and bioavailability and to maintain the activity, the biocompatible material to be linked to the insulin analogue having reduced insulin receptor binding affinity may include various bio-

compatible materials, for example, polyethylene glycol, fatty acids, 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.

[78] When polyethylene glycol (PEG) is used as a carrier, Ambrx's 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 also be employed, but is not limited thereto. It is apparent that any technology 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 included.

[79] When albumin is used as a carrier, a technology of directly linking albumin or an albumin fragment to the insulin analogue via 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.

[80] 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 also be 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 of increasing in-vivo half-life using an Fc fragment may also be 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 an insulin analogue may be 1:1 or 1:2, but is not limited thereto.

[81] 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 also be included in the present invention. The peptide or protein fragment to be used may be ELP (Elastin-like polypeptide) composed of repeating units of a particular amino acid combination, and versartis's Xten technology of using an artificial polypeptide PEG is also employed in the present invention. Zealand's SIP (Structure inducing probe) technology of increasing in-vivo half-life using multi-Lysine is also employed, and Prolor's CTP fusion technology is also employed. Use of transferrin which is known to increase in-vivo stability, fibronectin which is a connective tissue component, or derivatives thereof may also be 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 is also 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.

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

[83] 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 to 100 kDa, or 10 Da to 100 kDa. Further, one or more of polyethylene glycol may be linked thereto.

[84] The polyethylene glycol may be linked to one or more amino acid residues selected from the group consisting of a N-terminal amino acid and a 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.

[85]

[86] 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 also be included.

[87] For example, the amino acid sequence may be a repeated sequence of a repeating unit (VPGXG, SEQ ID NO: 49)n, wherein X is selected from alanine, arginine, asparagine, aspartic acid, glutamic acid, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine and valine residues, and n is 1 to 500.

[88] 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, (AVGVP, SEQ ID NO: 56)n, (IPGVG, SEQ ID NO: 57)n, (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 also be included in the scope of the present invention (n=1 to 500).

[89]

[90] Abbreviations of the amino acids used herein are in accordance with the IUPAC-IUB nomenclature as follows.

[91] Alanine A Arginine R

[92] Asparagine N Aspartic acid D

[93] Cysteine C Glutamic acid E

[94] Glutamine Q Glycine G

[95] Histidine H Isoleucine I

[96] Leucine L Lysine K

[97] Methionine M Phenylalanine F

[98] Proline P Serine S

[99] Threonine T Tryptophan W

[100] Tyrosine Y Valine V

[101]

[102] Elastin, the 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 a sugar chain may be performed by adding sugar chain-modified amino acids during production in animal cells.

[103] In the present invention, binding of elastin, a water-soluble precursor of elastin or an elastin-like polypeptide to insulin showing reduced insulin RMC (receptor-mediated clearance) may be performed via a peptide bond by genetic recombination, or they may be produced separately and then bind to each other via a non-peptidyl polymer.

[104]

[105] The linker which is able to link between the insulin analogue having reduced insulin

receptor-binding affinity and the biocompatible material or carrier capable of increasing in-vivo half-life of the insulin analogue may be a peptide or a non-peptidyl polymer. The insulin analogue (X) of the present invention and the biocompatible material or carrier (F) may be linked via the linker (L) by a covalent bond, a non-covalent bond, or a combination thereof. Specifically, 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.

[106]

[107] 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.

[108] 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.

[109] 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, oligonucleotides 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.

[110] The peptidyl linker which is used in the fusion protein obtained by a conventional inframe fusion method has drawbacks in 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.

[111] Polyethylene glycol or an elastin-like peptide which is a carrier linked via the non-peptidyl polymer of the present invention may be one carrier or a combination of different types of carriers via the non-peptidyl polymer.

[112] 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.

[113] 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 a 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.

[114] 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.

[115] Such insulin analogue conjugate of the present invention maintains in-vivo activities of the conventional insulin, such as energy metabolism and sugar metabolism, and also increases blood half-life of the insulin analogue and markedly increases duration of in-vivo efficacy of the peptide, and therefore, the conjugate is useful in the treatment of diabetes.

[116]

[117] In still another aspect, the present invention provides a long-acting insulin formulation including the insulin analogue or the insulin analogue conjugate. The long-acting insulin analogue formulation may be a long-acting insulin analogue formulation having increased in-vivo duration and stability. The long-acting formulation may be a pharmaceutical composition for treating diabetes.

[118] In still another aspect, the present invention provides a long-acting formulation for preventing or treating diabetes including the insulin analogue or the insulin analogue conjugate. Further, in a specific embodiment of the present invention, the present invention provides a long-acting formulation for preventing or treating diabetes including the insulin analogue or the insulin analogue conjugate; and the insulinotropic

peptide or the derivative thereof.

[119]

[120] 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.

[121] 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.

[122] 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.

[123] As used herein, the term "prevention" means 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" means 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.

[124] 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 also be formulated into solutions, suspensions, tablets, pills, capsules and long-acting preparations.

[125]

[126] 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.

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

[128]

[129] In still another aspect, the present invention provides a method for treating insulin-related diseases, including the step of administering the insulin analogue, 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.

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

[131] The term "administration", as used herein, means 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 is able to 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 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.

[132] 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.

[133]

## Mode for the Invention

[134]

[135] 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.

[136]

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

[138] 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.

[139] Each of the amino acid sequences modified in A chain or B chain and 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; Analogue 2 has a substitution of alanine for isoleucine at position 2 of A chain; Analogue 3 has a substitution of alanine for tyrosine at position 19 of A chain; Analogue 4 has a substitution of alanine for glycine at position 8 of B chain; Analogue 5 has a substitution of alanine for glycine at position 23 of B chain; Analogue 6 has a substitution of alanine for phenylalanine at position 24 of B chain; Analogue 7 has a substitution of alanine for phenylalanine at position 25 of B chain; Analogue 8 has a substitution of glutamic acid for tyrosine at position 14 of A chain; and Analogue 9 has a substitution of asparagine for tyrosine at position 14 of A chain.

[140]

[141] [Table 1]

| Analogue | Modifed sequence |
|---|---|
| Analogue 1 | $A^{1}G \rightarrow A$ |
| Analogue 2 | $A^{2}I \rightarrow A$ |
| Analogue 3 | $A^{19}Y \rightarrow A$ |
| Analogue 4 | $B^{8}G \rightarrow A$ |
| Analogue 5 | $B^{23}G \rightarrow A$ |
| Analogue 6 | $B^{24}F \rightarrow A$ |
| Analogue 7 | $B^{25}F \rightarrow A$ |
| Analogue 8 | $A^{14}Y \rightarrow E$ |
| Analogue 9 | $A^{14}Y \rightarrow N$ |

[142] Primers for amplification of the insulin analogues of Table 1 are given in the following Table 2.

[143]

[144] [Table 2]

| Analog | Sequence | SEQ ID NO |
|---|---|---|
| Analog 1 | 5′ GGGTCCCTGCAGAAGCGTGCGATTGTGGAACAATGCTGT 3′<br>5′ ACAGCATTGTTCCACAATCGCACGCTTCTGCAGGGACCC 3′ | SEQ ID NO 1<br>SEQ ID NO 2 |
| Analog 2 | 5′ TCCCTGCAGAAGCGTGGCGCGGTGGAACAATGCTGTACC 3′<br>5′ GGTACAGCATTGTTCCACCGCGCCACGCTTCTGCAGGGA 3′ | SEQ ID NO 3<br>SEQ ID NO 4 |
| Analog 3 | 5′ CTCTACCAGCTGGAAAACGCGTGTAACTGAGGATCC 3′<br>5′ GGATCCTCAGTTACACGCGTTTTCCAGCTGGTAGAG 3′ | SEQ ID NO 5<br>SEQ ID NO 6 |
| Analog 4 | 5′ GTTAACCAACACTTGTGTGCGTCACACCTGGTGGAAGCT 3′<br>5′ AGCTTCCACCAGGTGTGACGCACACAAGTGTTGGTTAAC 3′ | SEQ ID NO 7<br>SEQ ID NO 8 |
| Analog 5 | 5′ CTAGTGTGCGGGGAACGAGCGTTCTTCTACACACCCAAG 3′<br>5′ CTTGGGTGTGTAGAAGAACGCTCGTTCCCCGCACACTAG 3′ | SEQ ID NO 9<br>SEQ ID NO 10 |
| Analog 6 | 5′ GTGTGCGGGGAACGAGGCGCGTTCTACACACCCAAGACC 3′<br>5′ GGTCTTGGGTGTGTAGAACGCGCCTCGTTCCCCGCACAC 3′ | SEQ ID NO 11<br>SEQ ID NO 12 |
| Analog 7 | 5′ TGCGGGGAACGAGGCTTCGCGTACACACCCAAGACCCGC 3′<br>5′ GCGGGTCTTGGGTGTGTACGCGAAGCCTCGTTCCCCGCA 3′ | SEQ ID NO 13<br>SEQ ID NO 14 |
| Analog 8 | 5′-CCAGCATCTGCTCCCTCGAACAGCTGGAGAACTACTG-3′<br>5′-Cagtagttctccagctgttcgagggagcagatgctgg-3′ | SEQ ID NO 15<br>SEQ ID NO 16 |
| Analog 9 | 5′-CAGCATCTGCTCCCTCAACCAGCTGGAGAACTAC-3′<br>5′-Gtagttctccagctggttgagggagcagatgctg-3′ | SEQ ID NO 17<br>SEQ ID NO 18 |

[145]

[146] PCR for insulin analogue amplification using the primers of Table 2 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 DNA fragments obtained under the conditions as intracellular inclusion bodies, each of them was inserted into pET22b vector, and the expression vectors thus obtained 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.

[147]

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

[149]

[150] [Table 3]

| Analog | Sequence | | SEQ ID NO |
|---|---|---|---|
| Analog 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 |
| Analog 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 |
| Analog 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 |

[151]

| | | | |
|---|---|---|---|
| Analog 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 |
| Analog 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 |
| Analog 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 |

[152]

| | | | |
|---|---|---|---|
| Analog 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 |
| Analog 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 |
| Analog 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 |

[153]

[154] **Example 2: Expression of recombinant insulin analogue**

[155] 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. Transformation 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 dispensed to a cryotube, and stored at -140°C. These samples were used as cell stocks for production of the recombinant fusion proteins.

[156] 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 to 16 hours. When an OD600 value reached 5.0 or higher, the cultivation 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% 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, IPTG was introduced at a final concentration of 500 μM. After introduction, culture was further performed for about 23 to 25 hours. After terminating the culture, recombinant strains were harvested using a centrifuge, and stored at -80°C until use.

[157]

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

[159] 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 re-suspended 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 re-suspended 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 re-suspended in distilled water, followed by centrifugation in the same manner. The pellet thus obtained was re-suspended 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.

[160]

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

[162] 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.

[163]

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

[165] 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 a molar ratio of 1000 and carboxypeptidase B corresponding to a molar ratio of 2000 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 reduce pH to 3.5.

[166]

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

[168] 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.

[169]

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

[171] 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.

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

[173]

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

[175]

[176] [Table 4]

| Analogue | Chain | Protein sequence (mature form) | SEQ ID NO: |
|---|---|---|---|
| 1 | A | **Ala** Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn | 37 |
| | B | 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 | 47 |
| 2 | A | Gly **Ala** Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn | 38 |
| | B | 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 | 47 |
| 3 | A | Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn **Ala** Cys Asn | 39 |
| | B | 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 | 47 |
| 4 | A | Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn | 46 |
| | B | 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 | 40 |
| 5 | A | Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn | 46 |
| | B | 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 | 41 |
| 6 | A | Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn | 46 |
| | B | 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 | 42 |
| 7 | A | Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn | 46 |
| | B | 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 | 43 |
| 8 | A | Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu **Glu** Gln Leu Glu Asn Tyr Cys Asn | 44 |
| | B | 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 | 47 |
| 9 | A | Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu **Asn** Gln Leu Glu Asn Tyr Cys Asn | 45 |

[177]

| | | | |
|---|---|---|---|
| | B | 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 | 47 |

[178]

[179] In Table 4, amino acid positions which are specifically modified in the insulin analogues, based on the native insulin, are marked in bold. Further, formation of disulfide bonds between cysteine at position 6 of A chain and cysteine at position 11 of B chain, between cysteine at position 7 of A chain and cysteine at position 7 of B chain, and between cysteine at position 20 of A chain and cysteine at position 19 of B chain in the native insulin and insulin analogue sequences was confirmed.

[180]

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

[182] 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 BIA evaluation software. In this regard, the model used was 1:1 Langmuir binding with baseline drift.

[183]

[184] [Table 5]

| Name | $k_a$ (1/Ms, $X10^5$) | $k_d$ (1/s, $X10^{-3}$) | $K_D$ (nM) |
|---|---|---|---|
| Native insulin | 2.9 (100%) | 12.4 (100%) | 42.0 (100%) |
| Insulin Analogue (No. 8) | 1.78 (60.0%) | 12.9 (104.6%) | 73.4 (57.1%) |

[185] As a result, compared to native insulin, Insulin analogue (No. 8) was found to have a receptor binding affinity of 57.1% (Table 5). As such, it was confirmed that the insulin

analogues of the present invention have the remarkably reduced insulin receptor binding affinity, compared to the native insulin.

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

[187]

[188] **Example 9: Comparison of receptor-mediated clearance between native insulin and insulin analogues**

[189] In order to measure receptor-mediated clearance (RMC) of insulin analogues, mouse-derived 3T3-L1 adipogenic cell line was used to measure residual insulin. In this experiment, Insulin analogue 8 (SEQ ID NO: 34) was used as a representative insulin analogue.

[190] In detail, 3T3-L1 cells were subcultured in DMEM (Dulbeco's Modified Eagle's Medium, Gibco, Cat. No. 12430) supplemented with 10% NBCS (newborn calf serum) twice to three times a week. 3T3-L1 cells were suspended in a differentiation medium (DMEM supplemented with 10% FBS), and then inoculated in a 48-well plate at a density of 5 x $10^4$ cells per well, followed by incubation for 48 hours. For differentiation into adipocytes, 1 μg/ml of human insulin (Sigma, Cat. No. I9278), 0.5 mM IBMX (3-isobutyl-1-methylxanthine, Sigma, Cat. No. I5879), and 1 μM dexamethasone (Sigma, Cat. No. D4902) were mixed with the differentiation medium, and 250 μl thereof was added to each well after removing the existing medium. 48 hours later, the medium was replaced with the differentiation medium containing only 1 μg/ml of human insulin. Thereafter, while the medium was replaced with the differentiation medium containing only 1 μg/ml of human insulin every 48 hours, induction of differentiation into adipocytes was examined for 7 to 9 days.

[191] Subsequently, to measure residual insulin, native human insulin and a human insulin analogue (Insulin analogue 8, SEQ ID NO: 34) were diluted with serum-free DMEM medium at a concentration of 500 nM. The differentiated cells were washed with serum-free DMEM medium once, and then each 250 μl of the samples was added to the cells, followed by incubation in an incubator at 37°C, 5% $CO_2$. 0.5, 1, 2, 4, 8, 20, 24, 32, and 48 hours after incubation, the media were taken, and concentrations of insulin remaining in the media were analyzed using a human insulin ELISA kit (ALPCO, 80-INSHU-E10.1). The initial insulin concentration was taken as 100%, and a relative concentration at each time point of measurement was calculated. The measurement results are summarized in the following Table 6.

[192]

[193] [Table 6]

| | Residual amount in media | |
|---|---|---|
| Time (h) | Native insulin | Insulin analogue (Insulin analogue 8) |
| 0 | 100 | 100 |
| 0.5 | 94.5 ±5.5 | 93.0 ± 30.9 |
| 1 | 82.4 ± 3.4 | 91.9 ± 18.9 |
| 2 | 96.2 ± 0.8 | 101.6 ± 35.6 |
| 4 | 94.1 ± 9.2 | 122.1 ± 23.4 |
| 8 | 57.4 ± 10.7 | 116.0 ± 17.7 |
| 20 | 37.7 ± 7.1 | 101.3 ± 21.4 |
| 24 | 35.2 ± 15.1 | 96.8 ± 12.0 |
| 32 | 39.1 ± 1.3 | 103.2 ± 22.9 |
| 48 | 24.2 ± 5.9 | 102.9 ± 9.7 |

[194]

[195] As shown in Table 6, the results of measuring the residual amounts of insulin or an insulin analogue in media over time showed that 96.8% and 102.9% of an insulin analogue (Insulin analogue 8) remained whereas 35.2% and 24.2% of native insulin remained in the media at 24 and 48 hours. The initial concentration of the insulin analogue was maintained until 48 hours, whereas the residual amount of native insulin in the medium was gradually reduced over time (FIG. 1), suggesting that concentration change of the insulin analogue of the present invention is less affected by receptor-mediated clearance (RMC), compared to native insulin.

[196]

[197] **Example 10: Pharmacokinetics of native insulin and insulin analogue in nephrectomized animal model**

[198] Pharmacokinetics of native insulin and an insulin analogue were analyzed in

nephrectomized rats (SD rat, male, 6-week-old). As a control group of the nephrectomized rat, a sham-operated rat was used. In this experiment, Insulin analogue 8 (SEQ ID NO: 34) was used as a representative insulin analogue.

[199] In detail, 172 nM/kg of each of native insulin and an insulin analogue (Insulin analogue 8) was administered, and blood was collected over time to measure serum concentrations. The concentrations of native insulin and an insulin analogue remaining in serum at each time point were measured by enzyme linked immunosorbent assay (ELIS), and analyzed using a human insulin ELISA kit (ALPCO, 80-INSHU-E10.1) in accordance with the manufacturer's protocol.

[200] The results of analyzing pharmacokinetics of native insulin and an insulin analogue showed that serum concentration of an insulin analogue at each time point was increased both in the sham-operated group and the nephrectomized group, compared to native insulin (FIG. 2). Further, the maximum serum concentration and AUC (area under curve) of an insulin analogue were also higher than those of native insulin (FIG. 2), suggesting that concentration change of the insulin analogue is less affected by receptor-mediated clearance, compared to native insulin.

[201]

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

[203]

[204] 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.

[205]

# Claims

[Claim 1] An insulin analogue having a reduced insulin receptor-mediated clearance rate, compared to native insulin.

[Claim 2] The insulin analogue 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 insulin analogue of claim 1, wherein the insulin analogue is prepared by substitution of one or more amino acid(s) 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 in the native insulin with other amino acids, or by deletion thereof.

[Claim 4] The insulin analogue of claim 3, wherein the substitution to other amino acid(s) is substitution to amino acids 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 5] The insulin analogue of claim 1, wherein glutamic acid is substituted for tyrosine as an amino acid at position 14 of A chain of the native insulin.

[Claim 6] The insulin analogue of claim 5, wherein an alternation selected from the group consisting of substitution, addition, deletion, modification and combinations thereof further occurs in one or more amino acids, in addition to the above substitution.

[Claim 7] The insulin analogue of claim 1, wherein the insulin analogue is selected from the group consisting of an insulin analogue including A chain consisting of an amino acid sequence of SEQ ID NO: 37 and B chain consisting of an amino acid sequence of SEQ ID NO: 47; an insulin analogue including A chain consisting of an amino acid sequence of SEQ ID NO: 38 and B chain consisting of an amino acid sequence of SEQ ID NO: 47; an insulin analogue including A chain consisting of an amino acid sequence of SEQ ID NO: 39 and B chain consisting of an amino acid sequence of SEQ ID NO: 47; an insulin analogue including A chain consisting of an amino acid sequence of SEQ ID NO: 46 and B chain consisting of an amino acid sequence of SEQ ID NO: 40; an insulin analogue including A chain consisting of an

amino acid sequence of SEQ ID NO: 46 and B chain consisting of an amino acid sequence of SEQ ID NO: 41; an insulin analogue including A chain consisting of an amino acid sequence of SEQ ID NO: 46 and B chain consisting of an amino acid sequence of SEQ ID NO: 42; an insulin analogue including A chain consisting of an amino acid sequence of SEQ ID NO: 46 and B chain consisting of an amino acid sequence of SEQ ID NO: 43; an insulin analogue including A chain consisting of an amino acid sequence of SEQ ID NO: 44 and B chain consisting of an amino acid sequence of SEQ ID NO: 47; and an insulin analogue including A chain consisting of an amino acid sequence of SEQ ID NO: 45 and B chain consisting of an amino acid sequence of SEQ ID NO: 47.

[Claim 8] The insulin analogue of claim 7, wherein the insulin analogue is an insulin analogue comprising A chain consisting of an amino acid sequence of SEQ ID NO: 44 and B chain consisting of an amino acid sequence of SEQ ID NO: 47.

[Claim 9] The insulin analogue of claim 1, wherein the insulin analogue has a single-chain or a double-chain.

[Claim 10] An insulin analogue conjugate having a reduced insulin receptor-mediated clearance rate, compared to native insulin, wherein the conjugate has the following Chemical Formula:

[Chemical Formula]

X-La-F

wherein X is selected from the group consisting of the insulin analogue of any one of claims 1 to 9, and derivatives, fragments, and variants thereof,

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 11] The conjugate of claim 10, wherein the conjugate has a long-acting property capable of increasing in-vivo half-life.

[Claim 12] The conjugate of claim 10, wherein the linker is a non-peptidyl polymer.

[Claim 13] The conjugate of claim 12, 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 14] The conjugate of claim 13, 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, oligonucleotides and combinations thereof.

[Claim 15] The conjugate of claim 10, wherein polyethylene glycol has a molecular weight of 10 to 100,000 Da.

[Claim 16] The conjugate of claim 10, wherein X is linked to F via L by a covalent bond, a non-covalent bond or a combination thereof.

[Claim 17] The conjugate of claim 10, wherein the amino acid polymer is selected from the group consisting of an immunoglobulin fragment, elastin, a water-soluble precursor of elastin, and a polymer of repeating units of a part of the elastin protein sequence and combinations thereof.

[Claim 18] A long-acting insulin analogue formulation having increased in-vivo duration and stability, comprising the insulin analogue of any one of claims 1 to 9 or the insulin analogue conjugate of any one of claims 10 to 17.

[Claim 19] A long-acting formulation for preventing or treating diabetes, comprising the insulin analogue of any one of claims 1 to 9 or the insulin analogue conjugate of any one of claims 10 to 17.

[Claim 20] A long-acting formulation for preventing or treating diabetes, comprising the insulin analogue of any one of claims 1 to 9 or the insulin analogue conjugate of any one of claims 10 to 17; and an insulinotropic peptide or a derivative thereof.

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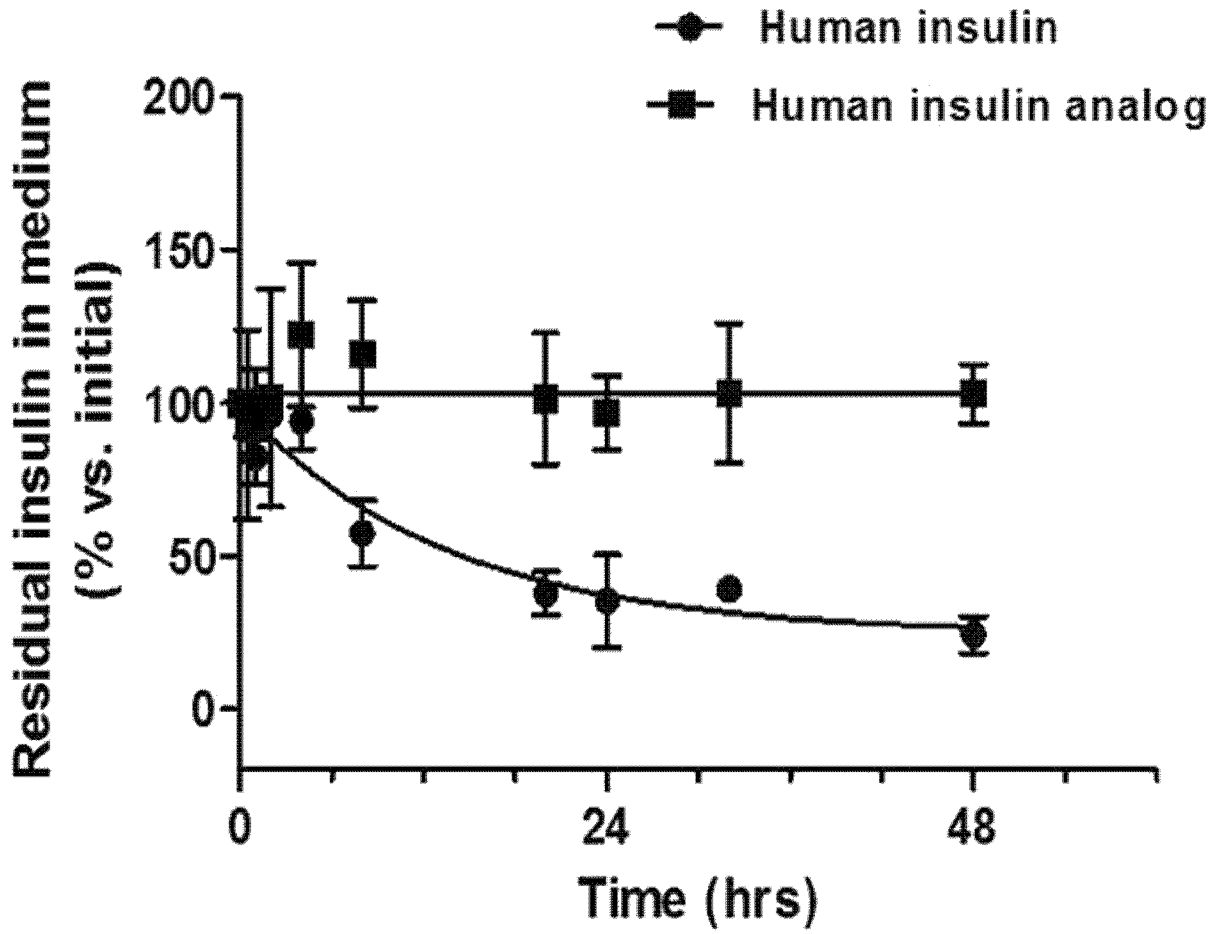
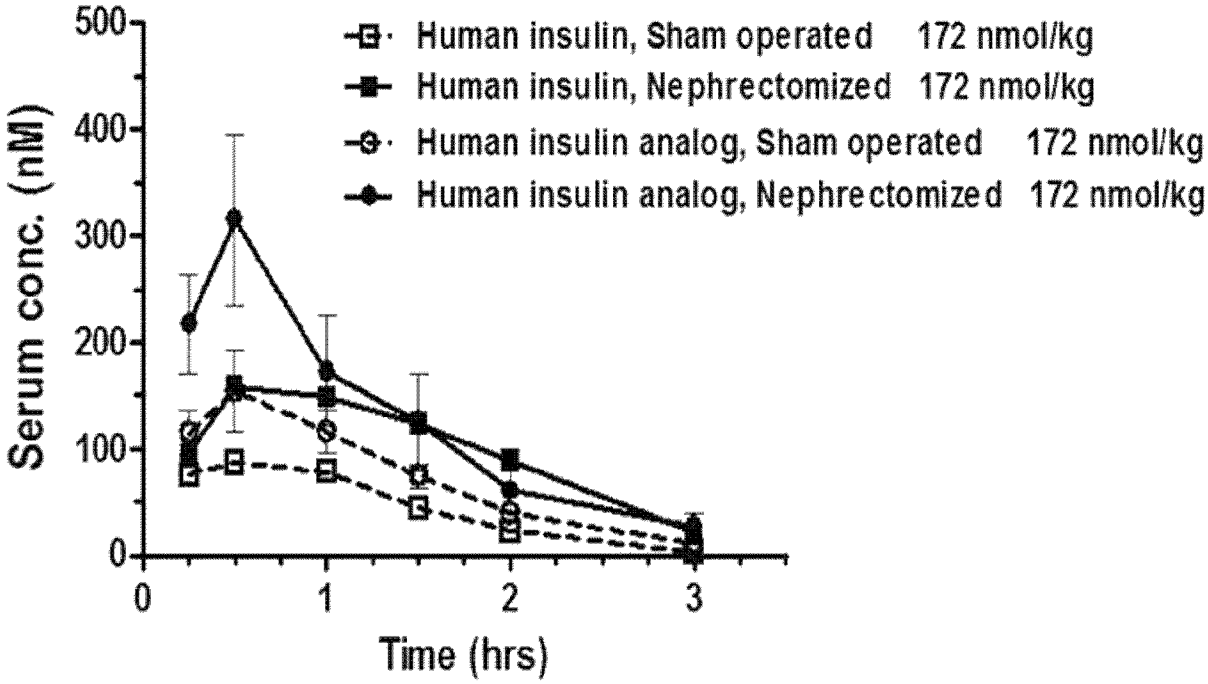
[Fig. 1]




[Fig. 2]



**INTERNATIONAL SEARCH REPORT**

International application No.
**PCT/KR2015/007182**

**A. CLASSIFICATION OF SUBJECT MATTER**

**C07K 14/62(2006.01)i, C12N 15/09(2006.01)i, C07K 19/00(2006.01)i, A61K 38/28(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 9/38; A61K 38/28; A61K 47/30; A61K 31/198; C12N 15/09; C07K 19/00

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: insulin analogue, receptor mediated clearance, conjugate, long acting, half life

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 2011-028813 A2 (CASE WESTERN RESERVE UNIVERSITY) 10 March 2011<br>See abstract; paragraphs [0009], [0010], [0032]; and claims 16-24. | 1-9 |
| Y | | 10-17 |
| Y | KR 10-2011-0134210 A (HANMI HOLDINGS CO., LTD.) 14 December 2011<br>See abstract; paragraphs [0026], [0042], [0055], [0072]; and claims 1-16. | 10-17 |
| A | DE MEYTS, 'The insulin receptor isoform A: a mitogenic proinsulin receptor?'<br>Endocrinology, Vol.153, No.5, pp.2054-2056 (2012)<br>See the whole document. | 1-17 |
| A | VIGNERI et al., 'Insulin and its analogs: actions via insulin and IGF receptors'<br>Acta Diabetologica, Vol.47, No.4, pp.271-278 (2010)<br>See the whole document. | 1-17 |
| A | ZAYKOV et al., 'Chemical synthesis of insulin analogs through a novel precursor'<br>ACS Chemical Biology, Vol.9, No.3, pp.683-691 (2013)<br>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:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 13 October 2015 (13.10.2015) | **14 October 2015 (14.10.2015)** |

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# INTERNATIONAL SEARCH REPORT

International application No.
**PCT/KR2015/007182**

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 18-20
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

**PCT/KR2015/007182**

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 2011-028813 A2 | 10/03/2011 | US 2012-0184488 A1 | 19/07/2012 |
| | | WO 2011-028813 A3 | 14/07/2011 |
| KR 10-2011-0134210 A | 14/12/2011 | KR 10-1330868 B1 | 18/11/2013 |