



US 20220056098A1

(19) **United States**

(12) **Patent Application Publication**
Lancaster et al.

(10) **Pub. No.: US 2022/0056098 A1**

(43) **Pub. Date: Feb. 24, 2022**

(54) **ULTRA-LONG ACTING INSULIN-FC FUSION PROTEIN AND METHODS OF USE**

(60) Provisional application No. 63/008,520, filed on Apr. 10, 2020.

(71) Applicant: **Akston Biosciences Corporation**,
Beverly, MA (US)

Publication Classification

(72) Inventors: **Thomas M. Lancaster**, Wenham, MA (US); **Todd C. Zion**, Marblehead, MA (US)

(51) **Int. Cl.**
C07K 14/62 (2006.01)

(52) **U.S. Cl.**
CPC **C07K 14/62** (2013.01); **A61K 38/00** (2013.01); **C07K 2319/30** (2013.01)

(21) Appl. No.: **17/527,486**

(22) Filed: **Nov. 16, 2021**

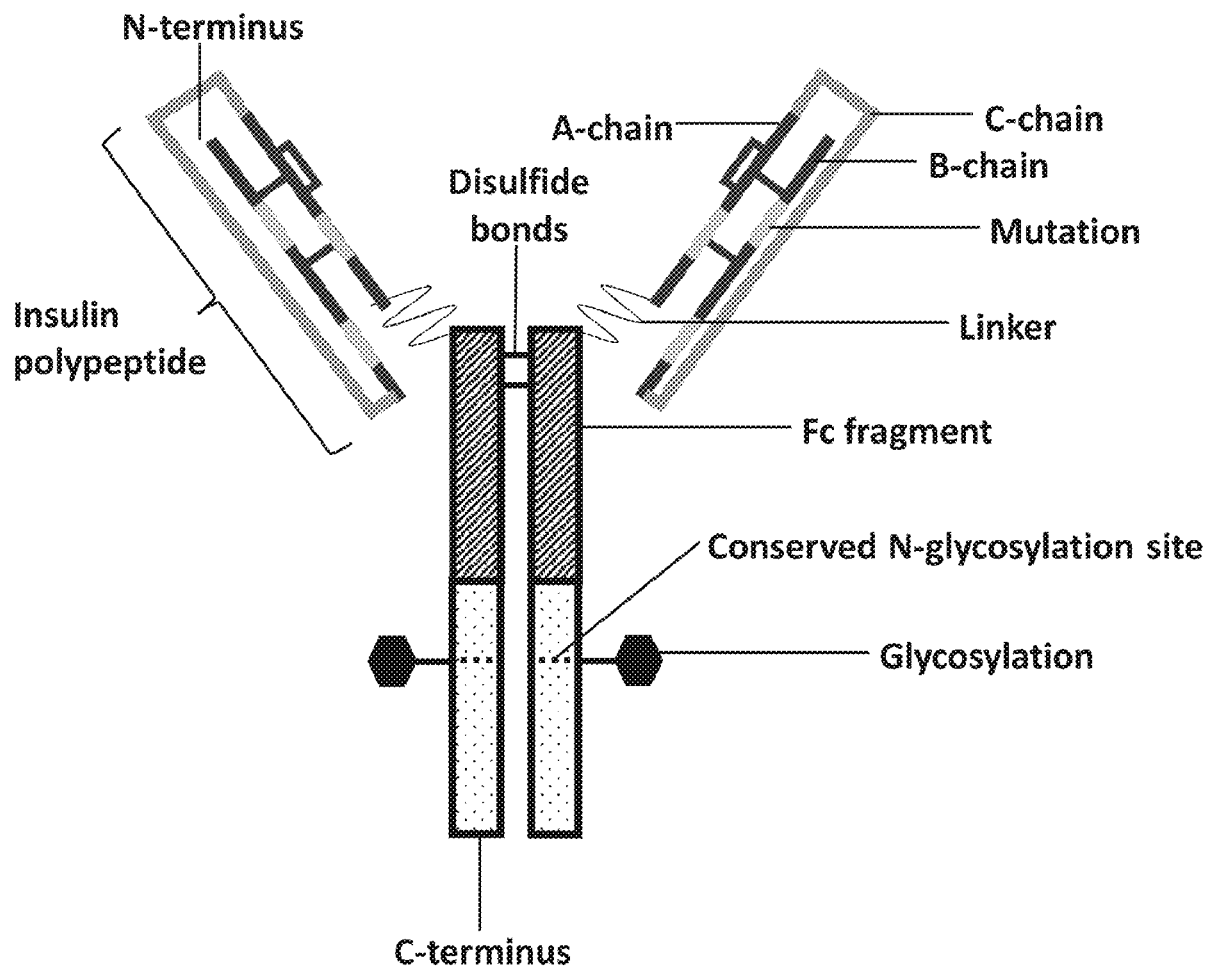
(57) **ABSTRACT**

Related U.S. Application Data

(63) Continuation of application No. 17/226,847, filed on Apr. 9, 2021, now Pat. No. 11,192,930.

The present disclosure relates to compositions of insulin-Fc fusion proteins and their use to treat feline diabetes.

Specification includes a Sequence Listing.



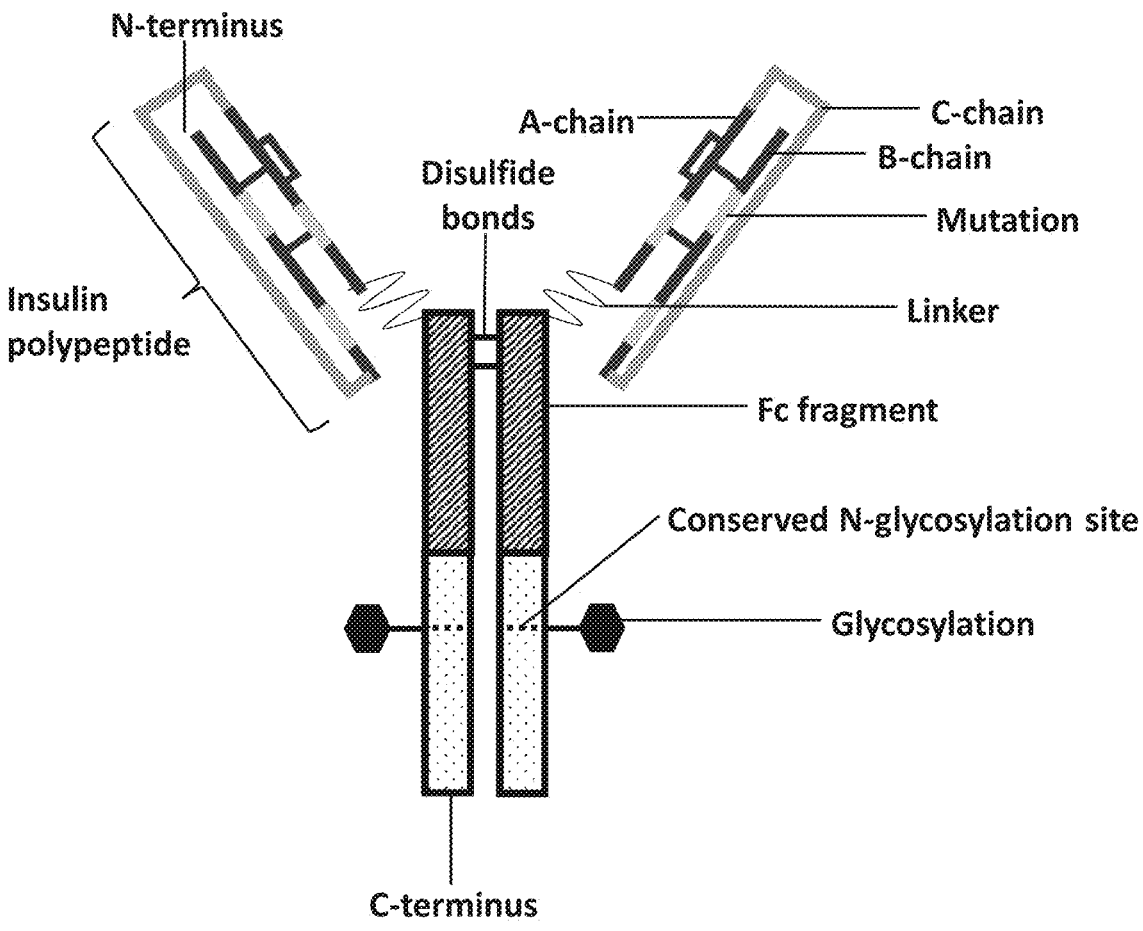


FIG. 1

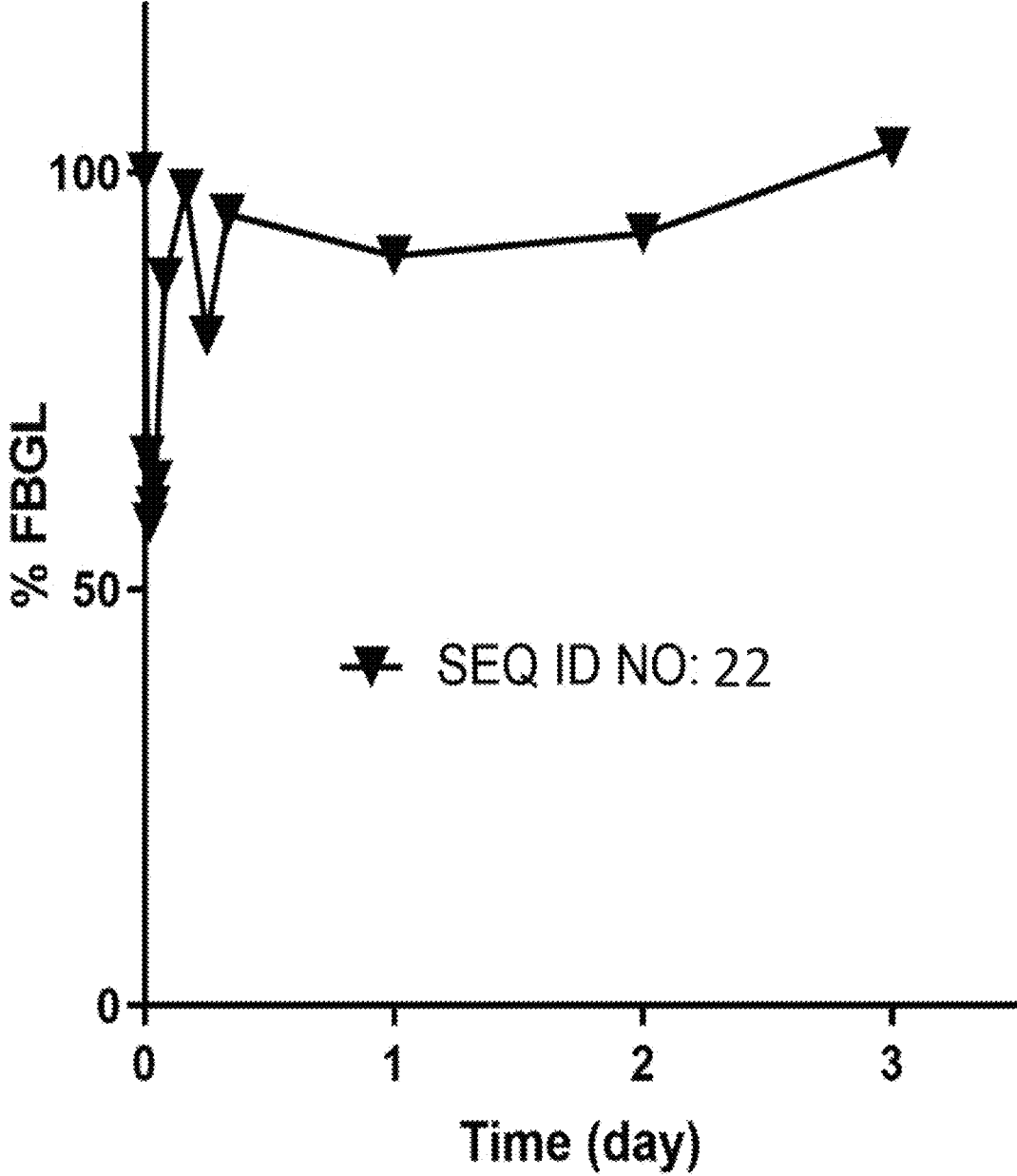


FIG. 2

SEQ ID NO: 22	FVNQHLCGSDLVEALALVCGERGFYTDPTGG-GPRRGIVEQCCHSICSLYQLENYCNGG	59
SEQ ID NO: 23	FVNQHLCGSDLVEALYLVCGERGFYTDPTGG-GPRRGIVEQCCHSICSLYQLENYCNGG	59
SEQ ID NO: 24	FVNQHLCGSHLVEALYLVCGERGFYTPKAGG-GPRRGIVEQCCTSICSLYQLENYCNGG	59
SEQ ID NO: 25	FVNQHLCGSHLVEALYLVCGERGFYTPKAAAAAAKGIIVEQCCTSICSLYQLENYCNGG ***** ,***** ***** ;. . . :***** *****	60
SEQ ID NO: 22	GGAGGGGRCTDTPPCPVPEPLGGPSVLIFFPKPKDILRITRTPEVTCVVLDLGGREDPEVQ	119
SEQ ID NO: 23	GGSGGGGRCTDTPPCPVPEPLGGPSVLIFFPKPKDILRITRTPEVTCVVLDLGGREDPEVQ	119
SEQ ID NO: 24	GGSGGGGRCTDTPPCPVPEPLGGPSVLIFFPKPKDILRITRTPEVTCVVLDLGGREDPEVQ	119
SEQ ID NO: 25	GGSGGGGRCTDTPPCPVPEPLGGPSVLIFFPKPKDILRITRTPEVTCVVLDLGGREDPEVQ ** :*****	120
SEQ ID NO: 22	ISWFVDGKEVHTAKTQSREQQFNGTYRVVSVLPIEHQDWTGKEFKCRVNHIDLPSPIER	179
SEQ ID NO: 23	ISWFVDGKEVHTAKTQSREQQFNGTYRVVSVLPIEHQDWTGKEFKCRVNHIDLPSPIER	179
SEQ ID NO: 24	ISWFVDGKEVHTAKTQSREQQFNGTYRVVSVLPIEHQDWTGKEFKCRVNHIDLPSPIER	179
SEQ ID NO: 25	ISWFVDGKEVHTAKTQSREQQFNGTYRVVSVLPIEHQDWTGKEFKCRVNHIDLPSPIER *****	180
SEQ ID NO: 22	TISKARGRAHKFSVYVLPSPKELSSSDTVSITCLIKDFYPPDIDVEWQSNQQEPPERKH	239
SEQ ID NO: 23	TISKARGRAHKFSVYVLPSPKELSSSDTVSITCLIKDFYPPDIDVEWQSNQQEPPERKH	239
SEQ ID NO: 24	TISKARGRAHKFSVYVLPSPKELSSSDTVSITCLIKDFYPPDIDVEWQSNQQEPPERKH	239
SEQ ID NO: 25	TISKARGRAHKFSVYVLPSPKELSSSDTVSITCLIKDFYPPDIDVEWQSNQQEPPERKH *****	240
SEQ ID NO: 22	RMTPPQLDEDGSYFLYSKLSVDKSRWQQGDPFTCAVMHETLQNHYTDLSSLHSPG	294
SEQ ID NO: 23	RMTPPQLDEDGSYFLYSKLSVDKSRWQQGDPFTCAVMHETLQNHYTDLSSLHSPG	294
SEQ ID NO: 24	RMTPPQLDEDGSYFLYSKLSVDKSRWQQGDPFTCAVMHETLQNHYTDLSSLHSPG	294
SEQ ID NO: 25	RMTPPQLDEDGSYFLYSKLSVDKSRWQQGDPFTCAVMHETLQNHYTDLSSLHSPG	295

FIG. 3

SEQ ID NO: 32	FVNQHLCGSDLVEALYLVCGERGFFYTDPTGGGPRRGIVEQCCHSICSLYQLENYCNGGG	60
SEQ ID NO: 34	FVNQHLCGSDLVEALYLVCGERGFFYTDPTGGGPRRGIVEQCCHSICSLYQLENYCNGGG	60
SEQ ID NO: 36	FVNQHLCGSDLVEALYLVCGERGFFYTDPTGGGPRRGIVEQCCHSICSLYQLENYCNGGG *****	60
SEQ ID NO: 32	GSGGGGGEGPKCFVPEIFGAPSVFIFPPKPKDLSISRTEVTCLVVDLGPDDSNVQITW	120
SEQ ID NO: 34	GSGGGGGEGPKCFVPEIFGAPSVFIFPPKPKDLSISRTEVTCLVVDLGPDDSNVQITW	120
SEQ ID NO: 36	GSGGGGGEGPKCFVPEIFGAPSVFIFPPKPKDLSISRTEVTCLVVDLGPDDSNVQITW *****	120
SEQ ID NO: 32	FVDNTEMHTAKTRPREEQFNSTYRVVSVLPILHQDWLKGKEFKCKVNSKSLPSAMERTIS	180
SEQ ID NO: 34	FVDNTEMHTAKTRPREEQFSSTYRVVSVLPILHQDWLKGKEFKCKVNSKSLPSAMERTIS	180
SEQ ID NO: 36	FVDNTEMHTAKTRPREEQFKSTYRVVSVLPILHQDWLKGKEFKCKVNSKSLPSAMERTIS *****	180
SEQ ID NO: 32	KAKGQPHEPQVYVLPPTQEELSENKSVTCLIRGFHPPDIAVEWEITGQPEPENNYQTTF	240
SEQ ID NO: 34	KAKGQPHEPQVYVLPPTQEELSENKSVTCLIRGFHPPDIAVEWEITGQPEPENNYQTTF	240
SEQ ID NO: 36	KAKGQPHEPQVYVLPPTQEELSENKSVTCLIRGFHPPDIAVEWEITGQPEPENNYQTTF *****	240
SEQ ID NO: 32	FQLSDGTYFLYSRLSVDRSHWQRGNTYTCVSVSHEALSHHTQKSLTQSPG	291
SEQ ID NO: 34	FQLSDGTYFLYSRLSVDRSHWQRGNTYTCVSVSHEALSHHTQKSLTQSPG	291
SEQ ID NO: 36	FQLSDGTYFLYSRLSVDRSHWQRGNTYTCVSVSHEALSHHTQKSLTQSPG *****	291

FIG. 4

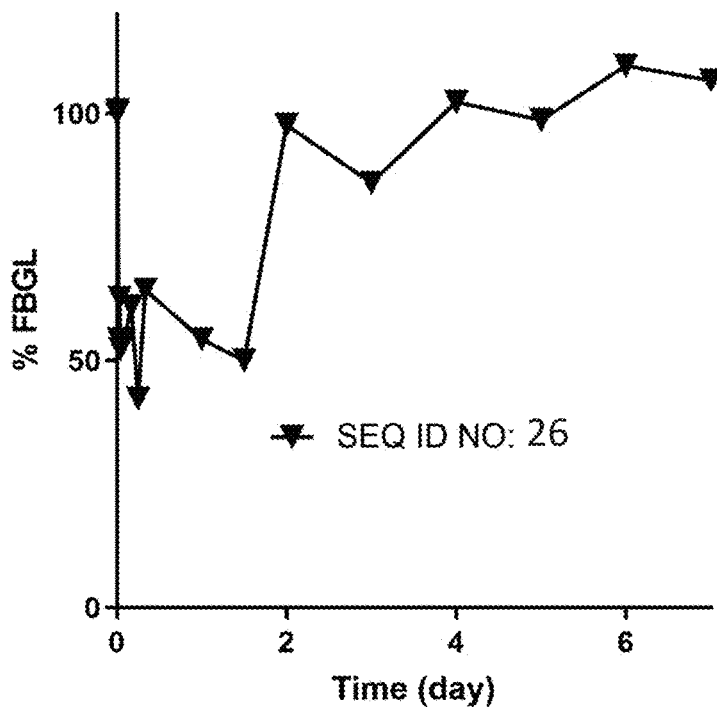


FIG. 5

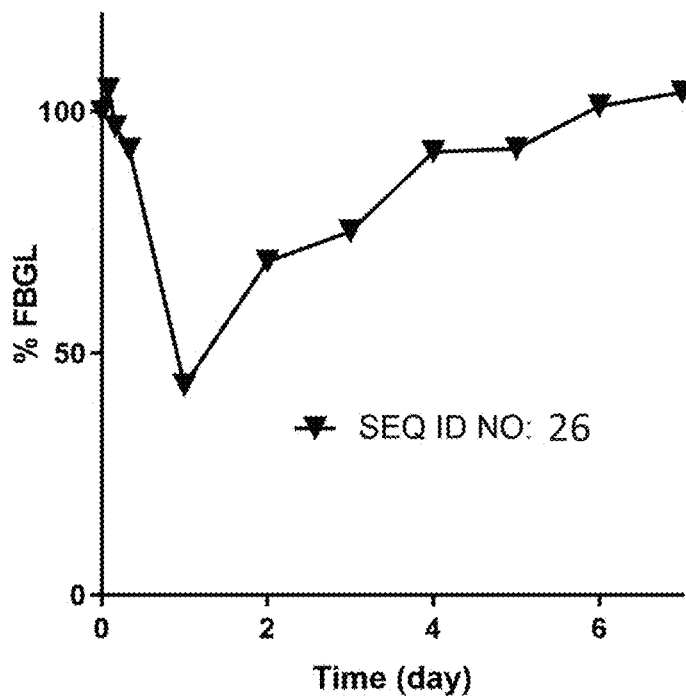


FIG. 6

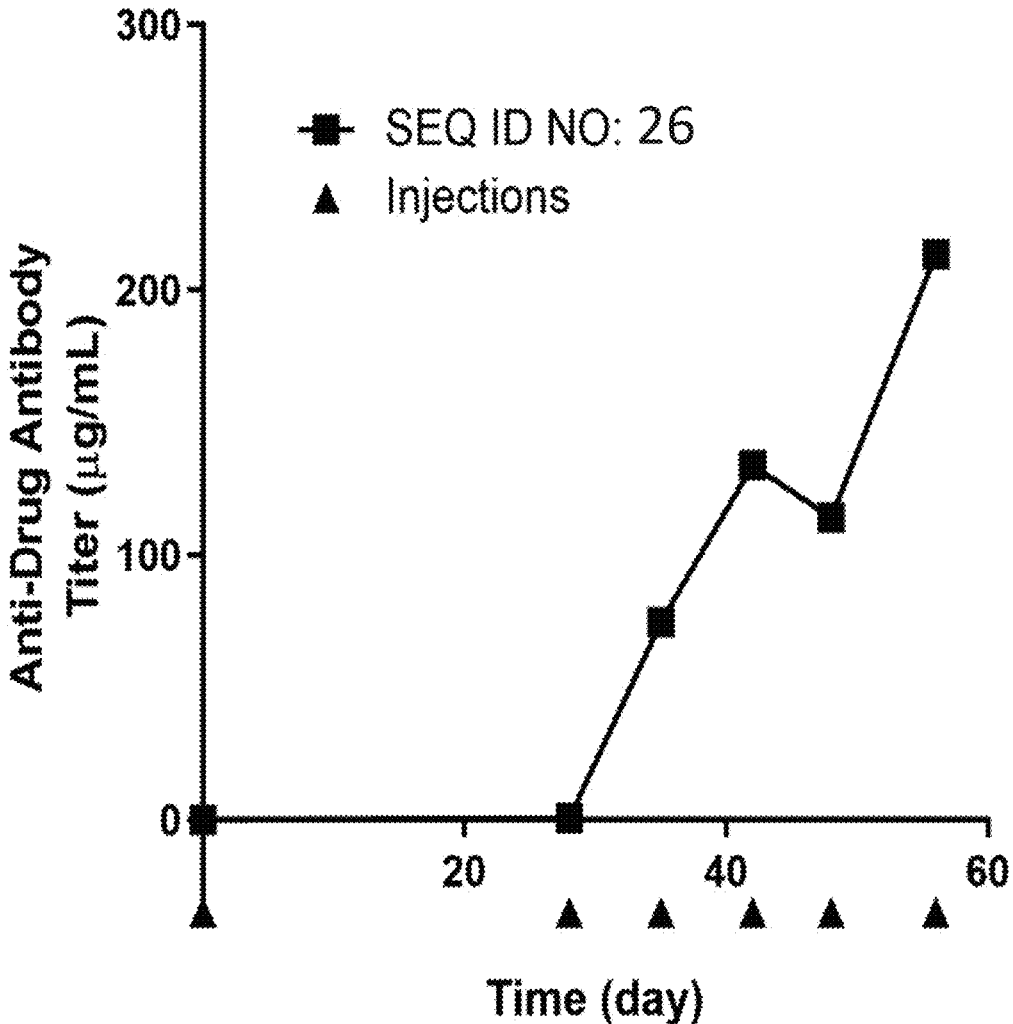


FIG. 7

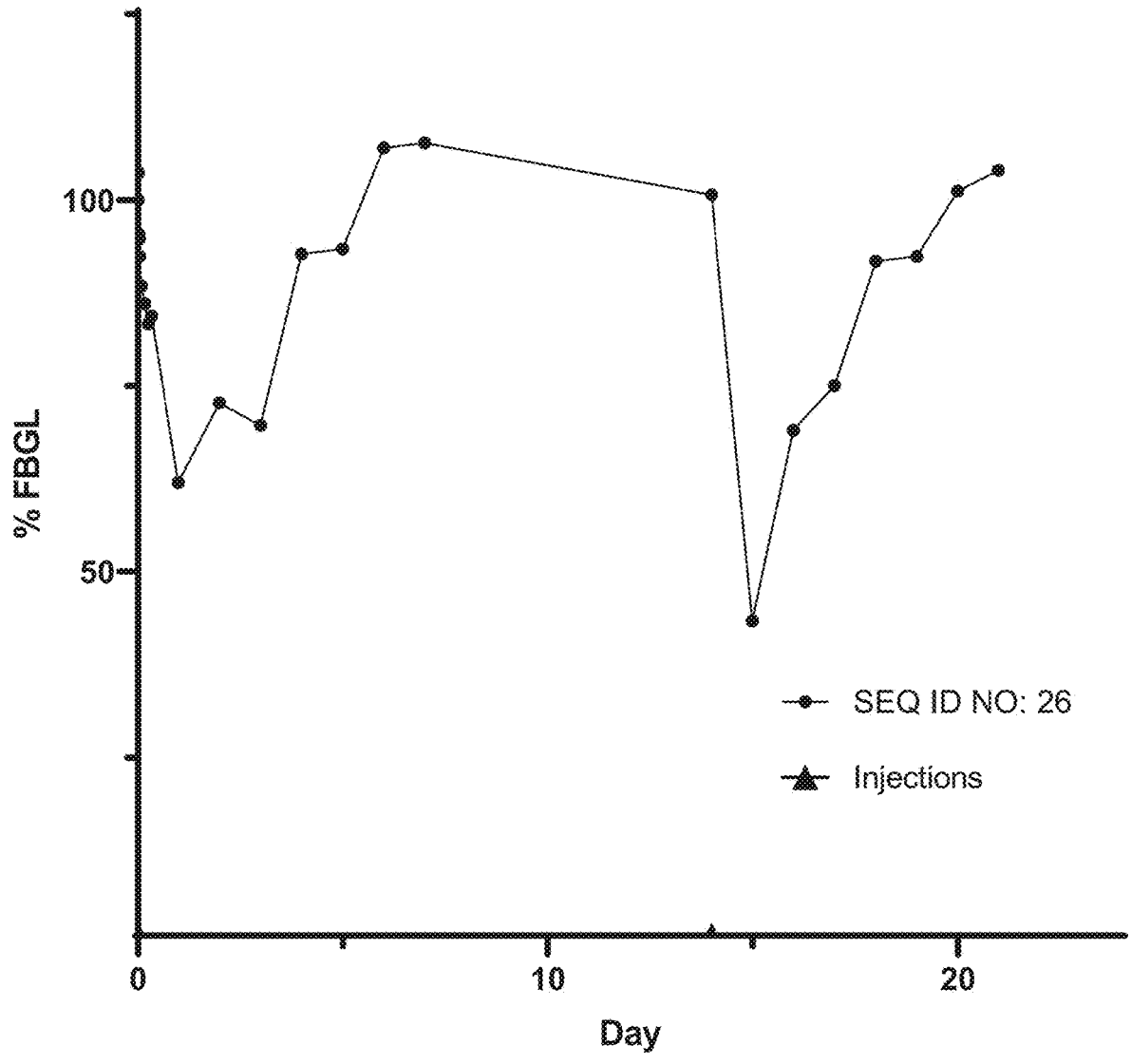


FIG. 8

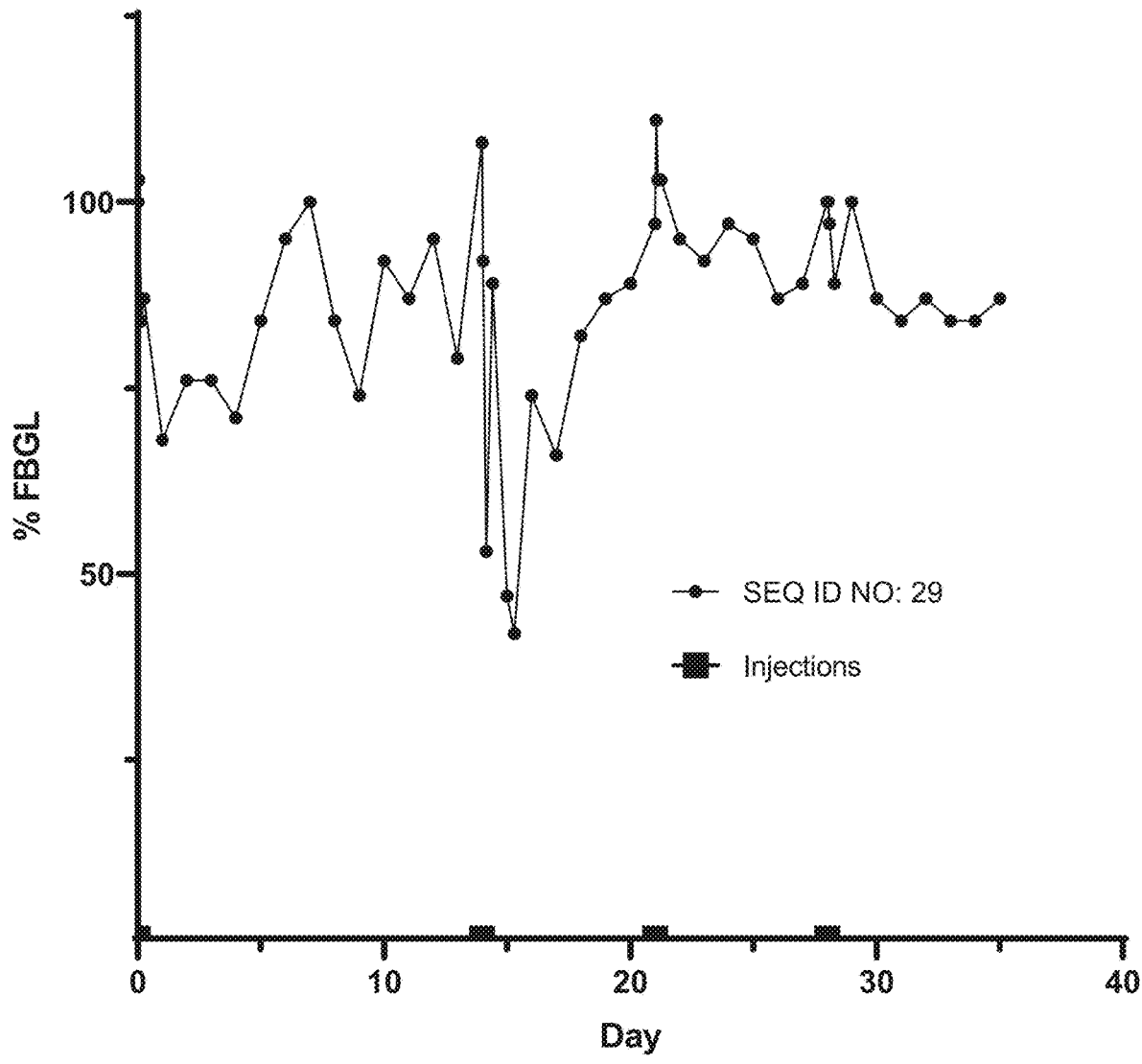


FIG. 9

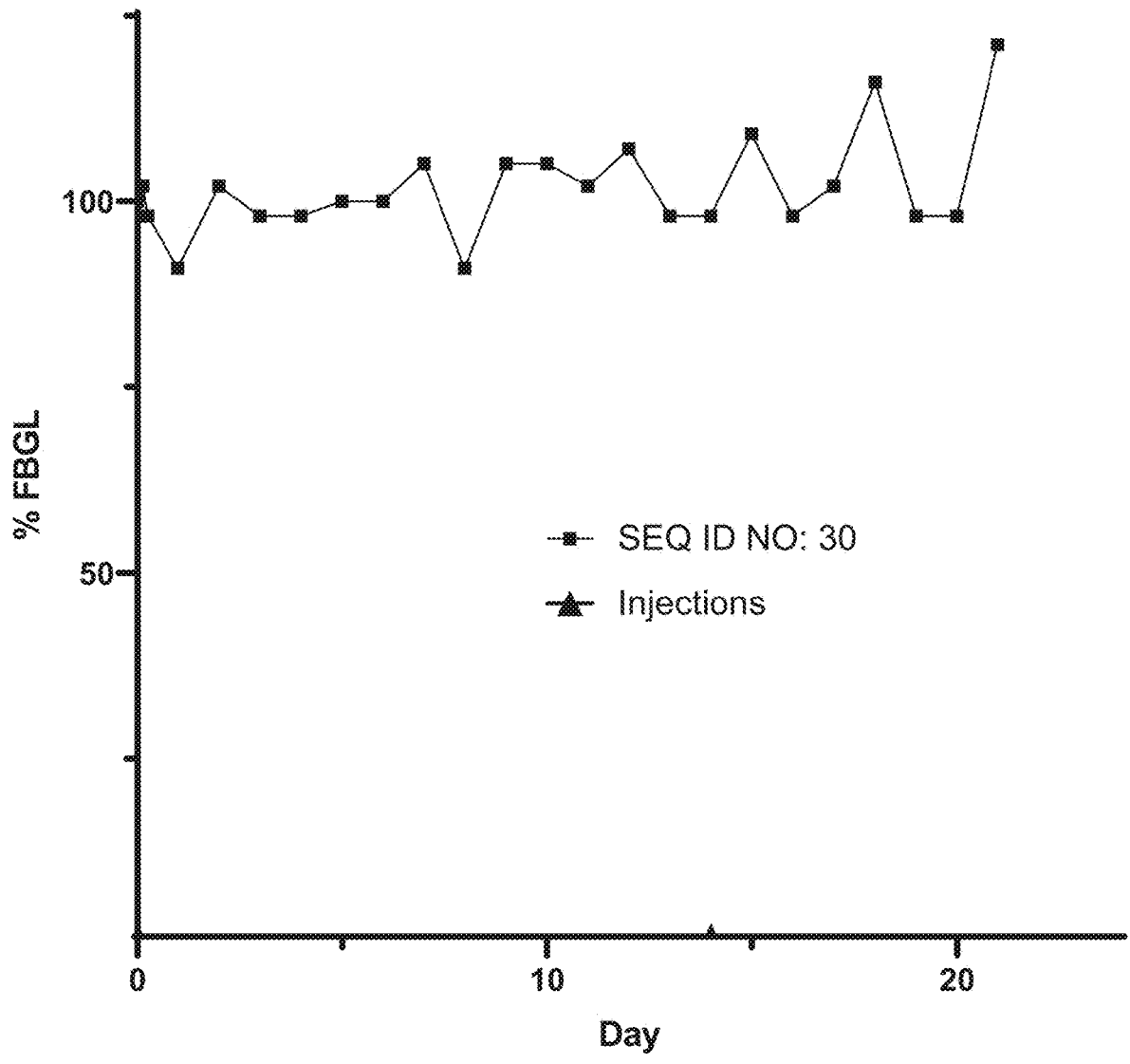


FIG. 10

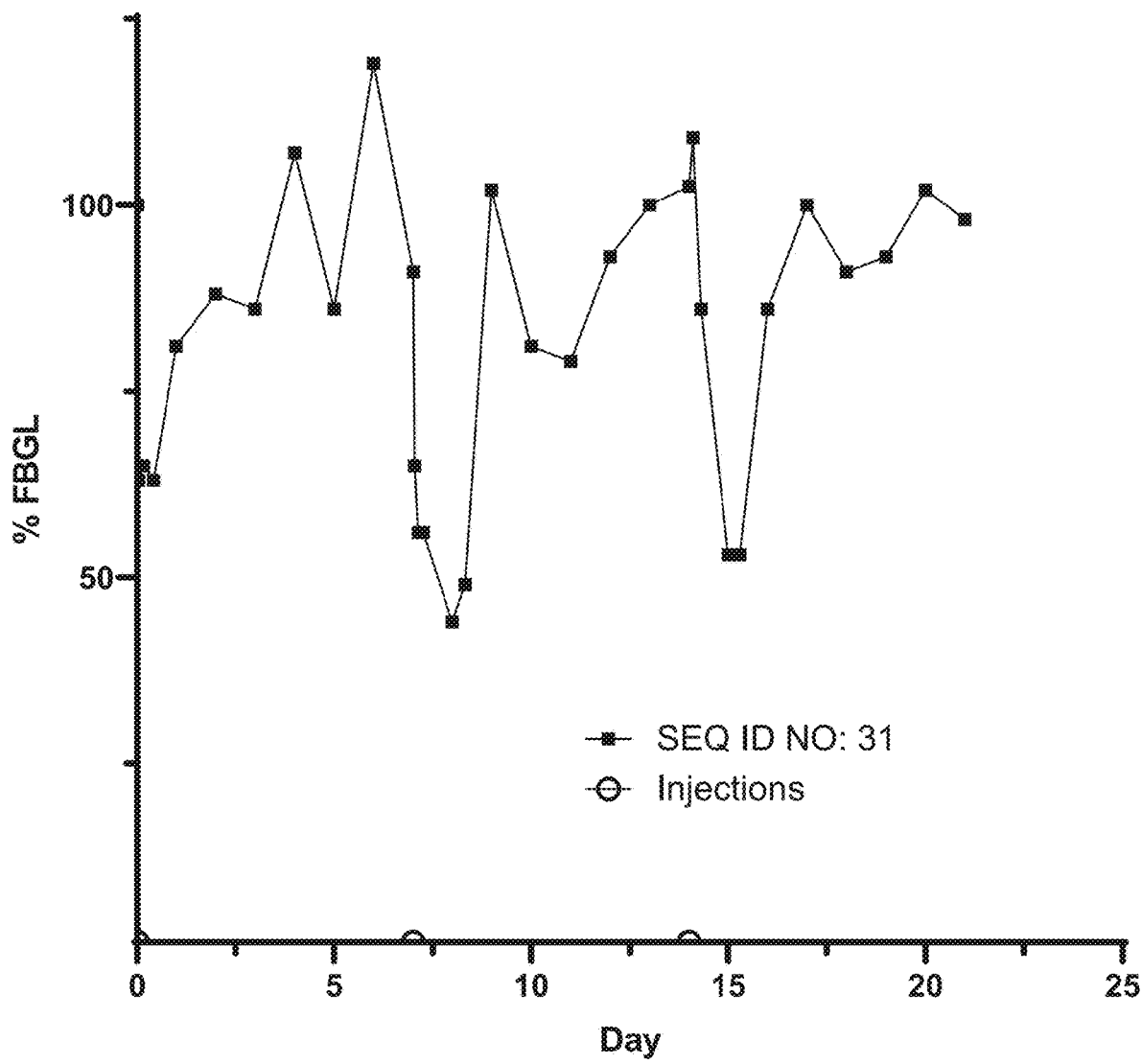


FIG. 11

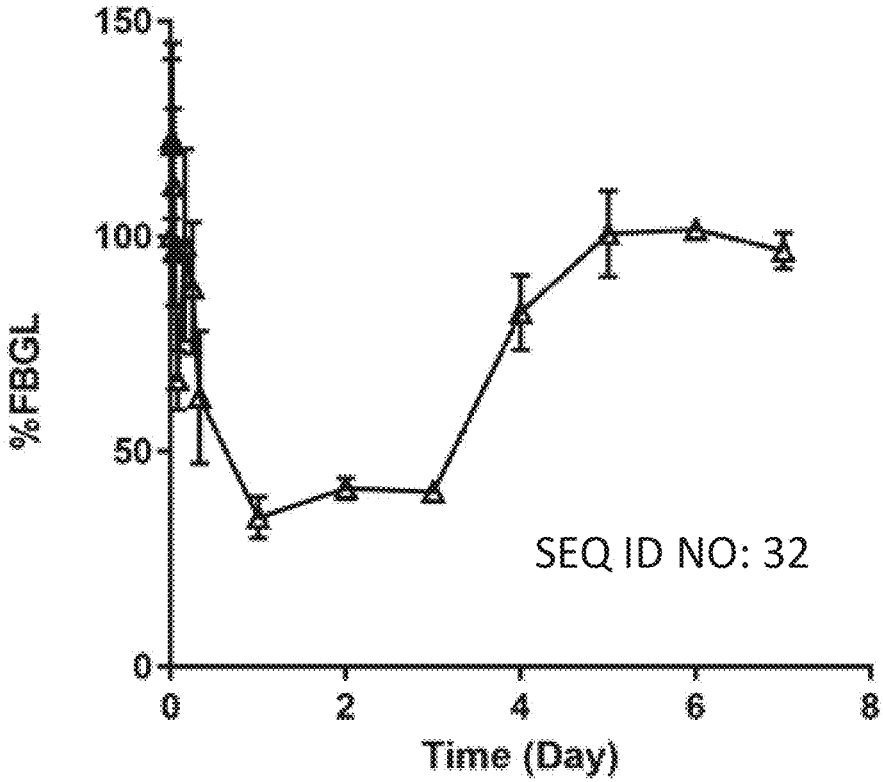


FIG. 12

ULTRA-LONG ACTING INSULIN-FC FUSION PROTEIN AND METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application is a continuation of U.S. patent application Ser. No. 17/226,847, filed Apr. 9, 2021, which claims the priority benefit of U.S. Provisional Patent Application Ser. No. 63/008,520, filed Apr. 10, 2020. The contents of the aforementioned patent applications are hereby incorporated herein by reference in their entirety.

SEQUENCE LISTING

[0002] The following application contains a sequence listing presenting in accordance with 37 C.R.F. 1.822. The sequence listing is entitled "SequenceListing031" submitted as an ASCII computer readable text file created on Nov. 4, 2021, as 65,564 bytes, which is incorporated by reference herein.

TECHNICAL FIELD

[0003] The present technology relates to compositions of insulin-Fc fusion proteins and their use to treat diabetes in companion animals, e.g., cats.

BACKGROUND

[0004] The following description of the background of the present technology is provided simply as an aid in understanding the present technology and is not admitted to describe or constitute prior art to the present technology.

[0005] Diabetes is a chronic condition characterized by an insulin deficiency and/or ineffective use of insulin. Diabetics that have an absolute deficiency of insulin are categorized as having type 1 or insulin-dependent diabetes mellitus (IDDM). Type 1 diabetics are thought to have a genetic predisposition combined with immunologic destruction of the insulin-producing β -cells of the pancreas. In comparison, diabetics that have the capability to still produce some insulin but have a relative deficiency due to insulin resistance or other dysfunction, are classified as having type 2 or non-insulin-dependent diabetes mellitus (NIDDM). Type 2 increased insulin production or increased insulin resistance is linked to genetic predisposition, obesity, and certain medications.

[0006] When a cat does not produce insulin or cannot use it normally, the cat's blood sugar levels elevate, resulting in hyperglycemia. Diabetic cats can fall under the type 1 (IDDM) or type 2 (NIDDM) classification. Generally, cats initially develop type 2 diabetes mellitus (insulin resistance), but by the time the disease is usually diagnosed by a veterinarian, it has progressed to type 1 (inflammatory disease in pancreas with significant loss of beta cell mass), and the cat is dependent on exogenous insulin. Some diabetic cats can be managed with dietary changes and oral medication, but the majority of diabetic cats need to receive insulin to maintain adequate regulation. Left untreated, it can lead to weight loss, loss of appetite, vomiting, dehydration, problems with motor function, coma, and even death. Cats that fall under the type 1 classification need daily shots to replace the missing insulin, but cats falling under the type 2 classification also stand to benefit from improved glycemic control through administration of additional exogenous insulin to help overcome their resistance to insulin.

[0007] Approximately 0.5% of cats in the United States have diabetes. Current diabetes therapies for cats include the use of insulin, such as ProZinc® (Boehringer Ingelheim Vetmedica, Duluth, Ga.) which is administered once or twice daily. The burden of frequent injections on owners often results in a lack of treatment regimen compliance and under dosing, leading to poor long-term health outcomes. In fact, the significant costs of insulin therapy and the practicality of dosing their pets up to 14 times per week leads to a significant percentage of owners to select euthanasia for their pets as an alternative to intensive management of diabetes. Therefore, there is a need for cost effective and less burdensome treatment options for this disease.

SUMMARY OF THE PRESENT TECHNOLOGY

[0008] In an aspect, the present disclosure provides a fusion protein comprising an insulin polypeptide and an Fc fragment, wherein the insulin polypeptide and the Fc fragment are connected by a linker such as a peptide linker, wherein the Fc fragment comprises the sequence:

(SEQ ID NO: 21)
 GEGPKCPVPEIPGAPSVFIFPPKPKDLSISRTPEVTCLVVDLGPDD
 SNVQITWFDNTEMHAKTRPREEQFKSYRVSVLPILHQDWLKGK
 EFKCKVNSKSLPSAMERTISKAKGQPHEPQVYVLPPTQEELSENKVS
 VTCLIKGFHPPDIAVEWEITGQPEPENNYQTTPPQLSDGTYFLYSR
 LSVDRSHWQRGNTYTCVSVSHEALHSHHTQKSLTQSPG.

[0009] In one embodiment, the present disclosure provides a fusion protein comprising an insulin polypeptide and an Fc fragment, wherein the insulin polypeptide and the Fc fragment are connected by a linker (e.g., peptide linker) comprising the sequence GGGGSGGGG (SEQ ID NO: 10).

[0010] In an embodiment, the present disclosure provides for a fusion protein comprising an insulin polypeptide and an Fc fragment, wherein the insulin polypeptide and the Fc fragment are connected by a linker (e.g., peptide linker), and wherein the fusion protein comprises the sequence

(SEQ ID NO: 36)
 FVNQHLCGSDLVEALYLVCGERGFFYTDPTGGGPRRGIVEQCCHSIC
 SLYQLENYCNNGGGSGGGGEGPKCPVPEIPGAPSVFIFPPKPKDLS
 SISRTPEVTCLVVDLGPDDSNVQITWFDNTEMHAKTRPREEQFKS
 TYRVSVLPILHQDWLKGKFKCKVNSKSLPSAMERTISKAKGQPHE
 PQVYVLPPTQEELSENKVSVTCLIKGFHPPDIAVEWEITGQPEPEN
 NYQTTPPQLSDGTYFLYSRSLVDRSHWQRGNTYTCVSVSHEALHSHHT
 QKSLTQSPG.

[0011] In embodiments, a fusion protein of the present disclosure comprises a dimer, wherein the dimer comprises two identical monomers bound together via disulfide bonds e.g., the fusion protein is a homodimer. In embodiments, the percentage homodimer of insulin-Fc fusion protein is greater than or equal to 90%.

[0012] In embodiments, the fusion proteins described herein are made using HEK293 cells, and the resulting homodimer titer after purification using Protein A beads or

a Protein A column is greater than 40 mg/L. In embodiments, the insulin receptor IC50 for the fusion proteins described herein is less than or equal to 5000 nM. In embodiments, the serum half-life of the fusion proteins described herein in the blood or serum of a target animal upon administration is longer than about 3 days. For the fusion proteins described herein, the time during which there is a statistically significant decrease in blood glucose level in a subject relative to a pre-dose level is longer than one of 2 hours, 6 hours, 9 hours, 12 hours, 18 hours, 1 day, 1.5 days, 2 days, 2.5 days, 3 days, 4 days, 5 days, 6 days, 7 days or longer.

[0013] In aspects, for the fusion proteins described herein, the NAOC after the first subcutaneous injection at a dose between 0.025 and 0.5 mg/kg/week in a target animal is greater than 150% FBGL·days·kg/mg. In embodiments, for the fusion proteins described herein, the ratio of the NAOC after the third weekly subcutaneous injection of the fusion proteins in the target animal to the NAOC after the first subcutaneous injection of the fusion protein in the target animal is greater than 0.50.

[0014] In aspects, fusion proteins as described herein are formulated as a pharmaceutical composition. In embodiments, in the pharmaceutical composition the fusion protein is present at a concentration of about 3 mg/mL or greater. In embodiments, the composition is suitable for subcutaneous administration.

[0015] In aspects, a method is described for lowering the blood glucose level of a cat, the method comprising administering a physiologically effective amount of a fusion protein as described herein or a pharmaceutical composition thereof to the cat. In embodiments, the cat is diagnosed with diabetes. In some embodiments, the fusion protein is administered subcutaneously. In some embodiments, the fusion protein is administered daily, twice weekly, or once weekly to the cat. In examples, the fusion protein is administered once weekly to the cat at a dose between 0.025 and 0.5 mg/kg/week. In aspects, a cell engineered to express a fusion protein as described herein. In examples, the cell is transfected with a nucleic acid encoding the fusion protein. In examples, the cell is a HEK293 cell or a CHO cell.

[0016] In an aspect, a cDNA encoding a fusion protein as described herein is described. In embodiments, the cDNA comprises the nucleic acid sequence encoding the insulin-Fc fusion protein of SEQ ID NO: 36 where the leader sequence is in bold:

(SEQ ID NO: 37)

ATGGAATGGAGCTGGGTCTTCTCTTCTTCCTGTCAAGTAACTGACTGG
TGTCCACTCCTTCGTGAACAGCACCTGTGCGGCTCCGACCTGGTGG
 AAGCTCTGTATCTCGTGTGCGGCGAGCGGGGCTTCTTACACCGAT
 CCCACTGGAGCGGTCCACGCAGAGGCATCGTGAACAGTGCTGCCA
 CTCCATCTGCTCCCTGTACCAGCTGGAAAACACTGCAATGGCGGAG
 GTGGTTCAGGAGGCGGTGGAGGTGAGGGCCCCAAGTGCCCCGTGCC
 GAGATTCCCGGTGCCCCAGCGTGTTCATCTTCCCCAAAACCCAA
 GGACACCTGAGCATCAGCAGGACCCCCGAGGTGACCTGCCTGGTGG
 TGGACCTGGGACCCGACGACAGCAACGTGCAGATCACCTGGTTCGTG
 GACAACCCGAGATGCACACCGCCAAAGACCAGGCCCTCGGGAGGAGCA

-continued

GTTCAGAGCACCTACAGGGTGGTGGAGCGTGCTGCCATCCTGCACC
 AGGACTGGCTGAAGGGCAAGGAGTTCAAGTGCAAGGTGAACAGCAAG
 AGCCTGCCAGCGCCATGGAGAGGACCATCAGCAAGGCCAAGGGTCA
 GCCCCACGAGCCCCAAGTGTACGTGCTTCCCCGACCCAGGAGGAGT
 TGAGCGAGAACAAAGTGAGCGTGACCTGCCTGATCAAGGGCTTCCAC
 CCTCCCGACATCGCCGTGGAGTGGGAGATCACCGGCCAGCCTGAGCC
 GGAAAATAACTACCAGACCACCCCTCCACAGCTGGACAGCGACGGCA
 CCTACTTCTTTACAGCAGGCTGTCTGTGGACCGAAGCCATTGGCAA
 AGGGGCAACACCTACACCTGCAGCGTGAGCCACGAGGCTCTGCACAG
 CCACCACACCCAGAAGTCTCTGACCCAGAGCCCCGGATAG.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] FIG. 1 shows a schematic representation of an exemplary insulin-Fc fusion protein homodimer.

[0018] FIG. 2 shows average % fasting blood glucose levels from Day 0 to Day 3 for N=3 dogs dosed intravenously on Day 0 at 0.2 mg/kg with the homodimer of SEQ ID NO: 22.

[0019] FIG. 3 illustrates a side-by-side sequence comparison of SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24 and SEQ ID NO: 25. "*" represents complete homology across all sequences at a given sequence position, while ":", "." or spaces refer to conservative, moderate, or very different amino acid mutations across the sequences at a given sequence position, respectively.

[0020] FIG. 4 illustrates a side-by-side sequence comparison of SEQ ID NO: 32, SEQ ID NO: 34, and SEQ ID NO: 36. "*" represents complete homology across all sequences at a given sequence position, while ":", "." or spaces refer to conservative, moderate, or very different amino acid mutations across the sequences at a given sequence position, respectively.

[0021] FIG. 5 shows average % fasting blood glucose levels from Day 0 to Day 7 for N=3 dogs dosed intravenously on Day 0 at 0.2 mg/kg with the homodimer of SEQ ID NO: 26.

[0022] FIG. 6 shows average % fasting blood glucose levels from Day 0 to Day 7 for N=6 dogs dosed subcutaneously on Day 0 at 0.33 mg/kg with the homodimer of SEQ ID NO: 26.

[0023] FIG. 7 shows the average anti-drug antibody titer ($\mu\text{g/mL}$) for N=3 dogs dosed subcutaneously on Day 0 (0.30 mg/kg), Day 28 (0.33 mg/kg), Day 35 (0.33 mg/kg), Day 42 (0.50 mg/kg), Day 49 (1.00 mg/kg) and Day 56 (1.00 mg/kg) with the homodimer of SEQ ID NO: 26.

[0024] FIG. 8 shows % fasting blood glucose levels from Day 0 to Day 21 for N=1 dog dosed subcutaneously on Day 0 and Day 14 with the homodimer of SEQ ID NO: 26.

[0025] FIG. 9 shows % fasting blood glucose levels from Day 0 to Day 35 for N=1 dog dosed subcutaneously on Day 0, Day 14, Day 21 and Day 28 with the homodimer of SEQ ID NO: 29.

[0026] FIG. 10 shows % fasting blood glucose levels from Day 0 to Day 21 for N=1 dog dosed subcutaneously on Day 0 and Day 14 with the homodimer of SEQ ID NO: 30.

[0027] FIG. 11 shows % fasting blood glucose levels from Day 0 to Day 21 for N=1 dog dosed subcutaneously on Day 0, Day 7 and Day 14 with the homodimer of SEQ ID NO: 31.

[0028] FIG. 12 shows average % fasting blood glucose levels from Day 0 to Day 7 for a cat dosed with the homodimer of SEQ ID NO: 32.

DETAILED DESCRIPTION

[0029] An insulin treatment that requires less frequent dosing (e.g., once-weekly injections) would be less burdensome on the owners, leading to better compliance, fewer instances of euthanasia, and better outcomes for the pets. As disclosed herein, proposed ultra-long acting insulin treatments for veterinary clinical use comprise an insulin-Fc fusion protein making use of an Fc fragment to prolong their action in vivo. An insulin-Fc fusion protein suitable for an ultra-long acting treatment for diabetes should meet various design goals. An insulin-Fc fusion protein configuration suitable for an ultra-long acting treatment for diabetes in cats should be manufacturable in mammalian cells, for example human embryonic kidney (HEK, e.g., HEK293) cells, with an acceptable titer of the desired homodimer product (e.g., greater than 40 mg/L homodimer titer from transiently transfected HEK cells, greater than 75 mg/L from transiently transfected HEK cells, greater than 100 mg/L from transiently transfected HEK cells, etc.). Only candidates with a homodimer titer of greater than 40 mg/L from transiently transfected HEK cells are considered useful in the present invention, because experience has demonstrated that homodimer titers less than this level will not likely result in commercial production homodimer titers in Chinese hamster ovary (CHO) cells that meet the stringently low manufacturing cost requirements for veterinary products.

[0030] In addition, the molecule must bind the insulin receptor with an appreciable affinity (e.g., IC₅₀ less than 5000 nM, IC₅₀ less than 4000 nM, IC₅₀ less than 3000 nM, IC₅₀ less than 2500 nM, etc.) as measured in the 4° C. IM-9 insulin receptor binding assay. Based on experience, only molecules exhibiting insulin receptor activity IC₅₀ values less than 5000 nM are deemed likely to exhibit the requisite bioactivity in the target species. The molecule must also demonstrate sustained bioactivity in vivo (e.g., demonstrate glucose lowering activity greater than about 2 hours, 6 hours, 9 hours, 12 hours, 18 hours, 1 day, 1.5 days, 2 days, 2.5 days, 3 days, 4 days, 5 days, 6 days, 7 days, or longer) to justify less frequent dosing. The molecule must also demonstrate prolonged system residence time in the target animal (e.g., the serum half-life must be greater than 3 days, or longer). The bioactive potency and duration of the bioactivity may be quantitatively represented by calculating the area over the percent fasting blood glucose (% FBGL) curve normalized to a given dose in mg/kg (NAOC) with units of % FBGL·days·kg/mg as described in Example 15. The NAOC increases with a greater drop in % FBGL, which is the case where the molecule demonstrates increased bioactivity, and when the % FBGL takes longer to return to 100%, which is the case where the insulin-Fc fusion protein demonstrates increased duration of action. To be useful as described herein, a molecule must demonstrate a sufficiently high NAOC value (e.g., preferably NAOC greater than 150% FBGL·days·kg/mg, more preferably NAOC greater than 200% FBGL·days·kg/mg, and even more preferably NAOC greater than 250% FBGL·days·kg/mg). Based on

experience, at NAOC values greater than 150% FBGL·days·kg/mg, the dose requirements in the target species will be sufficiently low to reach an acceptable treatment cost.

[0031] There are insulin-Fc fusion proteins known that make use of a human Fc region to prolong their action in vivo. However, insulin-Fc fusion proteins with a human Fc, when used in cats, develop antibodies that neutralize their action. Replacing a human Fc with a feline IgG Fc isotype would help to prevent unwanted immunogenicity which could result in the creation of neutralizing anti-drug antibodies.

[0032] There are three feline Fc isotypes: IgG1a (SEQ ID NO: 17), IgG1b (SEQ ID NO: 18), and IgG2 (SEQ ID NO: 19) as shown below.

(SEQ ID NO: 17)
DCPKCPPPEMLGGPSIFIFPPKPKDTLSISRTEVPTCLVVDLGPDDSDVQITWFDNTQVYTAKTSPREEQFNSTYRVVSVLPILHQDWLKGKEFKCKVNSKSLPSPIERTISKAKGQPHQPQVYVLPQAEEELSRNKVSVTCLIKSFHPDDIAVEWEITGQPEPENNYRTTPPQLDSDGTYFVYSKLSVDRSHWQRGNTYTCVSHALHSHHTQKSLTQSPG

(SEQ ID NO: 18)
DCPKCPPPEMLGGPSIFIFPPKPKDTLSISRTEVPTCLVVDLGPDDSDVQITWFDNTQVYTAKTSPREEQFNSTYRVVSVLPILHQDWLKGKEFKCKVNSKSLPSPIERTISKDKGQPHQPQVYVLPQAEEELSRNKVSVTCLIEGFYPSDIAVEWEITGQPEPENNYRTTPPQLDSDGTYFLYSRLSVDRSRWQRGNTYTCVSHALHSHHTQKSLTQSPG

(SEQ ID NO: 19)
GEGPKCPVPEIPGAPSVFIFPPKPKDTLSISRTEVPTCLVVDLGPDDSNVQITWFDNTEMTAKTRPREEQFNSTYRVVSVLPILHQDWLKGKEFKCKVNSKSLPSAMERTISKAKGQPHQPQVYVLPPTQEELSENKSVTCLIKGFHPDDIAVEWEITGQPEPENNYQTTPPQLDSDGTYFLYSRLSVDRSHWQRGNTYTCVSHALHSHHTQKSLTQSPG

[0033] Replacing the human Fc fragment of an insulin-Fc fusion protein with a feline IgG2 isotype would be most preferable due to its lack of Fc(γ) effector function in felines (much like the human IgG2 isotype).

[0034] A feline insulin-Fc fusion protein may comprise an insulin analog polypeptide as shown in SEQ ID NO: 4 with mutations on the B-chain and A-chain and comprising a C-chain of SEQ ID NO: 8.

(SEQ ID NO: 8)
GGGPRR

(SEQ ID NO: 4)
FVNQHLCGSDLEALYLVCGERGFFYTD

TGGGPRRGIVEQCCHSI CSLYQLNENYCN

[0035] The insulin analog polypeptide of SEQ ID NO: 4 is connected via a peptide linker of SEQ ID NO: 10 to a feline IgG2 Fc fragment of SEQ ID NO: 19.

(SEQ ID NO: 10)
GGGSGGGG

(SEQ ID NO: 19)
GEGPKCPVPEIPGAPSVFIFPPKPKDTLSISRTPEVTCLVVDLGPDD
SNVQITWFDNTEMTAKTRPREEQFNSTYRVVSVLPILHQDWLKGK
EFKCKVNSKSLPSAMERTISKAKGQPHEPQVYVLPPTQEELSENKVS
VTCLIKGFHPPDIAVEWEITGQPEPENNYQTTPQLSDSGTYFLYSR
LSVDRSHWQRGNTYTCSVSHEALHSHHTQKSLTQSPG

[0036] The resulting feline insulin-Fc fusion protein is shown below in SEQ ID NO: 32.

(SEQ ID NO: 32)
FVNQHLGSDLVEALYLVCGERGFFYTDPTGGGPRRGIVEQCCHSIC
SLYQLENYCNGGGGSGGGGEGPKCPVPEIPGAPSVFIFPPKPKDTL
SISRTPEVTCLVVDLGPDDSNVQITWFDNTEMTAKTRPREEQFNS
TYRVVSVLPILHQDWLKGKFKCKVNSKSLPSAMERTISKAKGQPHE
PQVYVLPPTQEELSENKVSVTCLIKGFHPPDIAVEWEITGQPEPEN
YQTTPQLSDSGTYFLYSRSLVDRSHWQRGNTYTCSVSHEALHSHHT
QKSLTQSPG

[0037] The feline insulin-Fc fusion protein of SEQ ID NO: 32 illustrates acceptable in vivo bioactivity in cats when administered subcutaneously, as is shown in FIG. 12. However, after several weekly treatments of SEQ ID NO: 32, the cats showed an immune response to the treatment. It was discovered that the cats had generated antibodies which were neutralizing to the action of the treatment in vivo. Further analysis revealed that these antibodies were targeting the insulin polypeptide at positions where amino acids had been mutated from natural insulin. More specifically, the amino acid at position 10 on the insulin B-chain had been mutated from His to Asp, and the amino acid at position 8 on the insulin A-chain had been mutated from Thr to His.

[0038] Without wishing to be bound by theory, it is believed that non-glycosylated insulin Fc-fusion proteins are less immunogenic than their counterpart insulin-Fc fusion proteins that are left in their natural glycosylated state. Although the antibodies generated in response to treatment with SEQ ID NO: 32 were targeted at the insulin polypeptide, producing a non-glycosylated insulin-Fc fusion protein was considered to have the potential to reduce the immunogenicity profile overall. It is known that mutating the Fc fragment at the natural glycosylation site found at position 140 from the N-terminus will prevent glycosylation of the molecule during expression from mammalian cells. It was therefore expected that a mutation from asparagine to serine at the natural glycan site found at position 140 from the N-terminus of the insulin Fc fusion protein of SEQ ID NO: 32 (resulting in SEQ ID NO: 34) while retaining the same insulin polypeptide and peptide linker, would result in an insulin-Fc fusion protein that retains acceptable in vivo bioactivity in cats when administered subcutaneously, but which has a significantly reduced immune response when repeatedly dosed.

[0039] The feline Fc fragment with the mutation from asparagine to serine at the natural glycan site is given in SEQ ID NO: 20.

(SEQ ID NO: 20)
GEGPKCPVPEIPGAPSVFIFPPKPKDTLSISRTPEVTCLVVDLGPDD
SNVQITWFDNTEMTAKTRPREEQFSSTYRVVSVLPILHQDWLKGK
EFKCKVNSKSLPSAMERTISKAKGQPHEPQVYVLPPTQEELSENKVS
VTCLIKGFHPPDIAVEWEITGQPEPENNYQTTPQLSDSGTYFLYSR
LSVDRSHWQRGNTYTCSVSHEALHSHHTQKSLTQSPG

(SEQ ID NO: 34)
FVNQHLGSDLVEALYLVCGERGFFYTDPTGGGPRRGIVEQCCHSIC
SLYQLENYCNGGGGSGGGGEGPKCPVPEIPGAPSVFIFPPKPKDTL
SISRTPEVTCLVVDLGPDDSNVQITWFDNTEMTAKTRPREEQFSS
TYRVVSVLPILHQDWLKGKFKCKVNSKSLPSAMERTISKAKGQPHE
PQVYVLPPTQEELSENKVSVTCLIKGFHPPDIAVEWEITGQPEPEN
YQTTPQLSDSGTYFLYSRSLVDRSHWQRGNTYTCSVSHEALHSHHT
QKSLTQSPG

[0040] An analogous compound for canines comprises the insulin polypeptide of SEQ ID NO: 5 and linker of SEQ ID NO: 9 with the native canine IgGB fragment of SEQ ID NO: 12.

(SEQ ID NO: 12)
DCPKCPAPEMLGGPSVFIFPPKPKDTLLIARTPEVTCVVVDLDPEDP
EVQISWFVDGKQMOTAKTQPREEQFNGTYRVVSVLPIGHQDWLKGKQ
FTCKVNNKALPSPIERTISKARGQAHQPSVYVLPSPREELSKNTVSL
TCLIKDFPPPIDVVEWQSNQQEPESKYRTTPQLDEDGSGYFLYSKL
SVDKSRWQRGDTFICAVMHEALHNYHTQESLSHSPG

[0041] The resulting canine insulin-Fc fusion protein is given below in SEQ ID NO: 26.

(SEQ ID NO: 26)
FVNQHLGSDLVEALALVCGERGFFYTDPTGGGPRRGIVEQCCHSIC
SLYQLENYCNGGGGAGGGDCPKCPAPEMLGGPSVFIFPPKPKDTLL
IARTPEVTCVVVDLDPEDPEVQISWFVDGKQMOTAKTQPREEQFNGT
YRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIERTISKARGQAHQPS
VYVLPSPREELSKNTVSLTCLIKDFPPPIDVVEWQSNQQEPESKY
RTTPQLDEDGSGYFLYSKLSVDKSRWQRGDTFICAVMHEALHNYHTQ
ESLSHSPG

[0042] If the natural glycan site found at the cNg position of SEQ ID NO: 26 has the asparagine to serine mutation, the resulting canine insulin-Fc fusion protein is SEQ ID NO: 29. This compound for canines comprises the same insulin polypeptides of SEQ ID NO: 5 and linker of SEQ ID NO: 9 with the canine IgGB fragment of SEQ ID NO: 15 (which has an asparagine to serine mutation at the natural glycan site found at the cNg position).

(SEQ ID NO: 15)
 DCPKCPAPEMLGGPSVFI FPPKPKD TLLIARTPEVTCVVVDLDPEDP
 EVQISWFDGKQMQTAKTQPREEQFSGTYRVVSVLP IGHQDWLK GKQ
 FTCKVNNKALPSPIERTISKARGQAHQPSVYVLPSPREELSKNTVSL
 TCLIKDFPPDIDVEWQSNQOQEPESKYRTPPQLDEDEGSYFLYSKL
 SVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG

[0043] The resulting canine insulin-Fc fusion protein is given below in SEQ ID NO: 29.

(SEQ ID NO: 29)
 FVNQHLGSDLVEALALVCGERGFYTDPTGGGPRRGIVEQCCHSIC
 SLYQLENYCNGGGGAGGGGDCPKCPAPEMLGGPSVFI FPPKPKD TLL
 IARTPEVTCVVVDLDPEDPEVQISWFDGKQMQTAKTQPREEQFSGT
 YRVVSVLP IGHQDWLK GKQFTCKVNNKALPSPIERTISKARGQAHQ
 SVYVLPSPREELSKNTVSLTCLIKDFPPDIDVEWQSNQOQEPESKY
 RTPPQLDEDEGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQ
 ESLSHSPG

[0044] The insulin-Fc fusion protein of SEQ ID NO: 29 shows acceptable in vivo bioactivity in dogs when administered subcutaneously but continues to generate antibodies targeting the insulin polypeptide after a few weekly treatments. This change from asparagine to serine at the N glycosylation site of the Fc fragment (cNg) to decrease its immunogenic potential did not eliminate the immune response that is targeted to the mutated portions of the insulin polypeptide.

[0045] Unexpectedly, it has been shown that a canine insulin-Fc fusion protein (SEQ ID NO: 31 below) with the same insulin polypeptide (SEQ ID NO: 5) and linker (SEQ ID NO: 9) and canine IgGB fragment but with a different mutation at the N glycosylation site of the Fc fragment (cNg) (e.g., asparagine to lysine) and the aspartic acid from the hinge region of the Fc fragment removed shows acceptable in vivo bioactivity in dogs when administered subcutaneously and does not generate antibodies even after several weekly treatments.

[0046] The canine IgGB fragment with an asparagine to lysine mutation at the N glycosylation site of the Fc fragment (cNg) and the aspartic acid from the hinge region of the Fc fragment removed is given in SEQ ID NO: 16.

(SEQ ID NO: 16)
 CPKCPAPEMLGGPSVFI FPPKPKD TLLIARTPEVTCVVVDLDPEDPE
 VQISWFDGKQMQTAKTQPREEQFGYRVVSVLP IGHQDWLK GKQFT
 CKVNNKALPSPIERTISKARGQAHQPSVYVLPSPREELSKNTVSLTC
 LIKDFPPDIDVEWQSNQOQEPESKYRTPPQLDEDEGSYFLYSKLSV
 DKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG

[0047] The resulting canine insulin-Fc fusion protein is given in SEQ ID NO: 31.

(SEQ ID NO: 31)
 FVNQHLGSDLVEALALVCGERGFYTDPTGGGPRRGIVEQCCHSIC
 SLYQLENYCNGGGGAGGGGDCPKCPAPEMLGGPSVFI FPPKPKD TLLI
 ARTPEVTCVVVDLDPEDPEVQISWFDGKQMQTAKTQPREEQFKGT
 YRVVSVLP IGHQDWLK GKQFTCKVNNKALPSPIERTISKARGQAHQ
 SVYVLPSPREELSKNTVSLTCLIKDFPPDIDVEWQSNQOQEPESKYR
 TTPPQLDEDEGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQ
 ESLSHSPG

[0048] It is therefore expected that a mutation from asparagine to lysine at the natural glycan site found at position 140 from the N-terminus in the analogous compound for cats (e.g., SEQ ID NO: 32 which is the compound with the same insulin polypeptide and feline IgG2 fragment which was shown to be immunogenic), i.e., mutating SEQ ID NO: 32 resulting in SEQ ID NO: 36, will similarly result in an insulin-Fc fusion protein with acceptable in vivo bioactivity in cats when administered subcutaneously, and which does not generate antibodies even after several weekly treatments.

(SEQ ID NO: 36)
 FVNQHLGSDLVEALYLVCGERGFYTDPTGGGPRRGIVEQCCHSIC
 SLYQLENYCNGGGGSGGGGEGPKCPVPEIPGAPSVFI FPPKPKD TLL
 SISRTPVTCVVVDLGPDDSNVQITWFDNTEMTAKTRPREEQFKS
 TYRVVSVLP I LHQDWLK GKKEFKCKVNSKSLPSAMERTISKAGQPHE
 PQVYVLPPTQEELSENKVSVTCLIKGPHPPDIAVEWEITGQPEPENN
 YQTPPQLDSGTYFLYSRLSVD RSHWQRGNITYTCSVSHEALHSHHT
 QKSLTQSPG.

Definitions

[0049] As used herein, the articles “a” and “an” refer to one or more than one, e.g., to at least one of the grammatical object of the article. The use of the words “a” or “an” when used in conjunction with the term “comprising” herein may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.”
[0050] As used herein, “about” and “approximately” generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements. Exemplary degrees of error are within 20 percent (%), typically, within 10%, and more typically, within 5% of a given range of values.

[0051] As used herein, an amount of a molecule, compound, conjugate, or substance effective to treat a disorder (e.g., a disorder described herein), “therapeutically effective amount,” or “effective amount” refers to an amount of the molecule, compound, conjugate, or substance which is effective, upon single or multiple dose administration(s) to a subject, in treating a subject, or in curing, alleviating, relieving or improving a subject with a disorder (e.g., a disorder described herein) beyond that expected in the absence of such treatment.

[0052] As used herein, the term “analog” refers to a compound or conjugate (e.g., a compound or conjugate as

described herein, e.g., insulin) having a chemical structure like that of another compound or conjugate but differing from it in at least one aspect.

[0053] As used herein, the term “antibody” or “antibody molecule” refers to an immunoglobulin molecule (Ig), immunologically active portions of an immunoglobulin (Ig) molecule, i.e., a molecule that contains an antigen binding site that specifically binds, e.g., immunoreacts with, an antigen. As used herein, the term “antibody domain” refers to a variable or constant region of an immunoglobulin. It is documented in the art that antibodies comprise several classes, for example IgA, IgM, or IgG in the case of mammals (e.g., felines). Classes of immunoglobulins can be further classified into different isotypes such as IgG1, IgG2, and IgG3, etc., or in other instances IgG1a, IgG1b, and IgG2. Those skilled in the art will recognize that immunoglobulin isotypes of a given immunoglobulin class will comprise different amino acid sequences, structures, and functional properties from one another (e.g., different binding affinities to Fc(gamma) receptors). “Specifically binds” or “immunoreacts with” means that the antibody reacts with one or more antigenic determinants of the desired antigen and has a lower affinity for other polypeptides, e.g., does not react with other polypeptides.

[0054] As used herein, the term “area-under-the-curve” or “AUC” refers to the integrated area under the % FBGL vs. time curve for a subject after a given dose of an insulin-Fc fusion protein configuration is administered. As used herein, the term “area-over-the-curve” or “AOC” is used as a measure of the biological potency of an insulin-Fc fusion protein configuration such that the AOC equals the difference between the total possible area under the % FBGL vs. time curve and the AUC value. As used herein, the “normalized area-over-the-curve,” “normalized AOC,” or “NAOC” is the AOC value divided by the actual dose of insulin-Fc fusion protein administered. As used herein, the term “normalized AOC ratio” or “NAOCR” is the ratio of the NAOC resulting from a particular administration of an insulin-Fc fusion protein to the NAOC resulting from the first administration of an insulin-Fc fusion protein in a series of administrations. The NAOCR thus provides a measure of the change in biological activity of an insulin-Fc fusion protein after repeated administrations.

[0055] As used herein, the term “bioactivity,” “activity,” “biological activity,” “potency,” “bioactive potency,” or “biological potency” refers to the extent to which an insulin-Fc fusion protein activates the insulin receptor and/or exerts a reduction in blood glucose levels in a target subject. As used herein, “in vitro activity” or “insulin receptor activity” refers to the affinity with which an insulin-Fc fusion protein binds to the insulin receptor and is typically measured by the concentration at which an insulin-Fc fusion protein displaces half of an insulin reference standard from the insulin receptor in a competitive binding assay (i.e., IC50). As used herein, “in vivo activity” refers to the extent and duration of reduction in a target subject’s fasting blood glucose level after administration of an insulin-Fc fusion protein.

[0056] As used herein, the term “biosynthesis,” “recombinant synthesis,” or “recombinantly made” refers to the process by which an insulin-Fc fusion protein is expressed within a host cell by transfecting the cell with a nucleic acid molecule (e.g., vector) encoding the insulin-Fc fusion protein (e.g., where the entire insulin-Fc fusion protein is encoded by a single nucleic acid molecule). Exemplary host

cells include mammalian cells, e.g., HEK293 cells or CHO cells. The cells can be cultured using standard methods in the art and the expressed insulin-Fc fusion protein may be harvested and purified from the cell culture using standard methods in the art.

[0057] As used herein, the term “cell surface receptor” refers to a molecule such as a protein, generally found on the external surface of the membrane of a cell and which interacts with soluble molecules, e.g., molecules that circulate in the blood supply. In some embodiments, a cell surface receptor may include a hormone receptor (e.g., an insulin hormone receptor or insulin receptor (IR)) or an Fc receptor which binds to an Fc fragment or the Fc region of an antibody (e.g., an Fc(gamma) receptor, for example Fc(gamma) receptor I, or an Fc neonatal receptor, for example FcRn). As used herein, “in vitro activity” or “Fc(gamma) receptor activity” or “Fc(gamma) receptor binding” or “FcRn receptor activity” or “FcRn binding” refers to the affinity with which an insulin-Fc fusion protein binds to the Fc receptor (e.g. Fc(gamma) receptor or FcRn receptor) and is typically measured by the concentration of an insulin-Fc fusion protein that causes the insulin-Fc fusion protein to reach half of its maximum binding (i.e., EC50 value) as measured on an assay (e.g., an enzyme-linked immunosorbent assay (ELISA) assay) using OD 450 nm values as measured on a microplate reader. Alternatively, the affinity with which an insulin-Fc fusion protein binds to the Fc receptor (e.g., Fc(gamma) receptor or FcRn receptor) is measured by the OD 450 nm value obtained on a microplate reader in an enzyme-linked immunosorbent assay (ELISA) assay at a given concentration of the insulin-Fc fusion protein.

[0058] As used herein, the term “fasting blood glucose level” or “FBGL” refers to the average blood glucose level in a target subject at the end of a period during which no food is administered and just prior to the time at which an insulin-Fc fusion protein is administered. As used herein, the term “percent fasting blood glucose level,” “% fasting blood glucose level,” or “% FBGL” refers to the ratio of a given blood glucose level to the fasting blood glucose level multiplied by 100.

[0059] As used herein, the term “immunogenic” or “immunogenicity” refers to the capacity for a given molecule (e.g., an insulin-Fc fusion protein of the present invention) to provoke the immune system of a target subject.

[0060] As used herein, the term “insulin reference standard” is any one of: (i) a naturally occurring insulin from a mammal (e.g., a human or dog); (ii) an insulin polypeptide that does not comprise an Fc fragment; or (iii) a standard of care insulin (e.g., a commercially available insulin).

[0061] As used herein, the term “monomer” refers to a protein or a fusion protein comprising a single polypeptide. In some examples, the “monomer” is a protein or a fusion protein, e.g., a single polypeptide, comprising an insulin polypeptide and an Fc fragment polypeptide, wherein the insulin polypeptide and Fc fragment polypeptide are joined by a peptide linker to form the single polypeptide. In some examples, the monomer is encoded by a single nucleic acid molecule.

[0062] As used herein, “N-terminus” refers to the start of a protein or polypeptide that is initiated by an amino acid containing a free amine group that is the alpha-amino group of the amino acid (e.g., the free amino that is covalently linked to one carbon atom that is located adjacent to a

second carbon atom, wherein the second carbon atom is part of the carbonyl group of the amino acid). As used herein, “C-terminus” refers to the end of a protein or polypeptide that is terminated by an amino acid containing a carboxylic acid group, wherein the carbon atom of the carboxylic acid group is located adjacent to the alpha-amino group of the amino acid.

[0063] As used herein, “pharmacodynamics” or “PD” generally refers to the biological effects of an insulin-Fc fusion protein in a subject. Specifically, herein the PD refers to the measure of the reduction in fasting blood glucose level over time in a subject after the administration of an insulin-Fc fusion protein.

[0064] As used herein, “pharmacokinetics” or “PK” generally refers to the characteristic interactions of an insulin-Fc fusion protein and the body of the subject in terms of its absorption, distribution, metabolism, and excretion. Specifically, herein the PK refers to the concentration of an insulin-Fc fusion protein in the blood or serum of a subject at a given time after the administration of the insulin-Fc fusion protein. As used herein, “half-life” refers to the time taken for the concentration of insulin-Fc fusion protein in the blood or serum of a subject to reach half of its original value as calculated from a first order exponential decay model for drug elimination. Insulin-Fc fusion proteins with greater “half-life” values demonstrate greater duration of action in the target subject.

[0065] The terms “sequence identity” “sequence homology” “homology” or “identical” in amino acid or nucleotide sequences as used herein describes that the same nucleotides or amino acid residues are found within the variant and reference sequences when a specified, contiguous segment of the nucleotide sequence or amino acid sequence of the variant is aligned and compared to the nucleotide sequence or amino acid sequence of the reference sequence. Methods for sequence alignment and for determining identity between sequences are known in the art, including the use of Clustal Omega, which organizes, aligns, and compares sequences for similarity, wherein the software highlights each sequence position and compares across all sequences at that position and assigns one of the following scores: an “*” (asterisk) for sequence positions which have a single, fully conserved residue, a “:” (colon) indicates conservation between groups of strongly similar properties with scoring greater than 0.5 in the Gonnet PAM 250 matrix, and a “.” (period) indicates conservation between groups of weakly similar properties with scoring less than or equal to 0.5 in the Gonnet PAM 250 matrix, a “-” (dash) indicates a sequence gap, meaning that no local homology exists within a particular set of comparisons within a certain range of the sequences, and an empty space “ ” indicates little or no sequence homology for that particular position across the compared sequences. With respect to optimal alignment of two nucleotide sequences, the contiguous segment of the variant nucleotide sequence may have additional nucleotides or deleted nucleotides with respect to the reference nucleotide sequence. Likewise, for purposes of optimal alignment of two amino acid sequences, the contiguous segment of the variant amino acid sequence may have additional amino acid residues or deleted amino acid residues with respect to the reference amino acid sequence. In some embodiments, the contiguous segment used for comparison to the reference nucleotide sequence or reference amino acid sequence will comprise at least 6, 10, 15, or 20 contiguous nucleotides, or

amino acid residues, and may be 30, 40, 50, 100, or more nucleotides or amino acid residues. Corrections for increased sequence identity associated with inclusion of gaps in the variant’s nucleotide sequence or amino acid sequence can be made by assigning gap penalties. Methods of sequence alignment are known in the art.

[0066] In embodiments, the determination of percent identity or “homology” between two sequences is accomplished using a mathematical algorithm. For example, the percent identity of an amino acid sequence is determined using the Smith-Waterman homology search algorithm using an affine 6 gap search with a gap open penalty of 12 and a gap extension penalty of 2, BLOSUM matrix 62. In embodiments, the percent identity of a nucleotide sequence is determined using the Smith-Waterman homology search algorithm using a gap open penalty of 25 and a gap extension penalty of 5. Such a determination of sequence identity can be performed using, for example, the DeCypher Hardware Accelerator from TimeLogic.

[0067] As used herein, the term “homology” is used to compare two or more proteins by locating common structural characteristics and common spatial distribution of, for instance, beta strands, helices, and folds. Accordingly, homologous protein structures are defined by spatial analyses. Measuring structural homology involves computing the geometric-topological features of a space. One approach used to generate and analyze three-dimensional (3D) protein structures is homology modeling (also called comparative modeling or knowledge-based modeling) which works by finding similar sequences since 3D similarity reflects 2D similarity. Homologous structures do not imply sequence similarity as a necessary condition.

[0068] As used herein, the term “subject” or “target subject” or “patient” or “patient subject” are intended to include felines. Exemplary feline subjects include cats having a disease or a disorder, e.g., diabetes or another disease or disorder described herein, or normal subjects.

[0069] As used herein, the term “titer” or “yield” refers to the amount of a fusion protein product (e.g., an insulin-Fc fusion protein described herein) resulting from the biosynthesis (e.g., in a mammalian cell, e.g., in a HEK293 cell or CHO cell) per volume of the cell culture. The amount of product may be determined at any step of the production process (e.g., before or after purification), but the yield or titer is always stated per volume of the original cell culture. As used herein, the term “product yield” or “total protein yield” refers to the total amount of insulin-Fc fusion protein expressed by cells and purified via at least one affinity chromatography step (e.g., Protein A or Protein G) and includes monomers of insulin-Fc fusion protein, homodimers of insulin-Fc fusion protein, and higher-order molecular aggregates of homodimers of insulin-Fc fusion protein. As used herein, the term “percent homodimer” or “% homodimer” refers to the proportion of a fusion protein product (e.g., an insulin-Fc fusion protein described herein) that is the desired homodimer. As used herein, the term “homodimer titer” refers to the product of the % homodimer and the total protein yield after Protein A purification step reported per volume of the cell culture.

[0070] As used herein, the terms “treat” or “treating” a subject having a disease or a disorder refer to subjecting the subject to a regimen, for example the administration of a fusion protein, such as a fusion protein described herein, such that at least one symptom of the disease or disorder is

cured, healed, alleviated, relieved, altered, remedied, ameliorated, or improved. Treating includes administering an amount effective to alleviate, relieve, alter, remedy, ameliorate, improve, or affect the disease or disorder, or the symptoms of the disease or disorder. The treatment may inhibit deterioration or worsening of a symptom of a disease or disorder.

Insulin-Fc Fusion Protein Components and Structure

[0071] The present disclosure relates to a composition of a fusion protein (i.e., an insulin-Fc fusion protein described herein comprises) comprising an insulin polypeptide linked via a peptide linker to a species-specific Fc fragment, and its use to treat diabetes in dogs. As used herein, the terms “fusion protein” and “insulin-Fc fusion protein” refer to a protein comprising more than one part, for example from different sources (different proteins, polypeptides, cells, etc.), that are covalently linked through peptide bonds. The insulin-Fc fusion proteins are covalently linked by (i) connecting the genes that encode for each part into a single nucleic acid molecule and (ii) expressing in a host cell (e.g., HEK or CHO) the protein for which the nucleic acid molecule encodes as follows: (N-terminus)-insulin polypeptide-linker-Fc fragment-(C-terminus). The fully recombinant synthesis approach is preferred over methods in which the insulin polypeptide and Fc fragments are synthesized separately and then chemically conjugated. The chemical conjugation step and subsequent purification process increase the manufacturing complexity, reduce product yield, and increase cost.

[0072] As used herein, the term “dimer” refers to a protein or a fusion protein comprising two polypeptides linked covalently. In embodiments, two identical polypeptides are linked covalently (e.g., via disulfide bonds) forming a “homodimer”. FIG. 1 diagrammatically represents an insulin-Fc fusion protein homodimer. The insulin polypeptide comprises a B-chain, a C-chain and an A-chain, and each insulin polypeptide is joined via a peptide linker to an Fc fragment. Two identical insulin-Fc fusion protein monomers are joined together via disulfide bonds to form an insulin-Fc fusion protein homodimer. The total number of disulfide bonds may be greater or less than the number shown in FIG. 1. The Fc fragment comprises a conserved asparagine (N)-glycosylation site, at which glycols may bond during recombinant manufacturing of the insulin-Fc fusion protein in mammalian cells. In embodiments, the homodimer is encoded by a single nucleic acid molecule, wherein the homodimer is made recombinantly inside a cell by first forming insulin-Fc fusion protein monomers and by then assembling two identical insulin-Fc fusion protein monomers into the homodimer upon further processing inside the cell.

[0073] As used herein, the terms “multimer,” “multimeric,” or “multimeric state” refer to non-covalent, associated forms of Fc fusion protein dimers that may be in equilibrium with Fc fusion protein dimers or may act as permanently aggregated versions of Fc fusion protein dimers (e.g., dimers of Fc fusion protein homodimers, trimers of Fc fusion protein homodimers, tetramers of Fc fusion protein homodimers, or higher order aggregates containing five or more Fc fusion protein homodimers). It may be expected that multimeric forms of Fc fusion proteins may have different physical, stability, or pharmacologic activities from that of the, e.g., insulin-Fc fusion protein homodimers.

Insulin Polypeptide

[0074] An insulin polypeptide may be, for example, an insulin or insulin analog produced by β -cells in the islets of Langerhans within the pancreas. Insulin functions by regulating the absorption of glucose from the blood. Upon a stimulus, such as increased protein and glucose levels, insulin is released from β -cells and binds to the insulin receptor (IR), initiating a signal cascade that affects many aspects of mammalian (e.g., human, feline, etc.) metabolism. Disruption of this process is directly related to several diseases, notably diabetes, insulinoma, insulin resistance, metabolic syndromes, and polycystic ovary syndrome.

[0075] Insulin analogs of the present disclosure may be related to the structure of insulin yet contain one or more modifications. In some embodiments, the insulin analog comprises at least one amino acid substitution, deletion, addition, or chemical modification relative to insulin, which may impact a particular feature or characteristic of the insulin-Fc fusion protein (e.g., insulin-Fc fusion protein described herein) configuration. For example, the modifications or alterations described herein may impact the structure, stability, pH sensitivity, bioactivity, or binding affinity of the insulin-Fc fusion protein to a cell surface receptor (e.g., an insulin hormone receptor). In some embodiments, an amino acid substitution, addition, deletion, or a chemical modification relative to insulin may affect the activity of the insulin or insulin analog relative to a reference standard. In some embodiments, the position of the amino acid sequence is referenced to the N-terminus of the insulin polypeptide, with position 1 being the first amino acid of the B-chain of the insulin polypeptide. In embodiments, the insulin or insulin analog is a three-segment peptide comprising elements of a B-chain, a C-peptide, and an A-chain. In embodiments, the insulin or insulin analog comprises a wild-type insulin B, C, and/or A chain, e.g., from a cat. In other embodiments, an insulin-Fc fusion protein described herein comprises an insulin polypeptide comprising a mutant insulin B-chain, C-peptide, and/or A-chain configuration to a cell surface receptor (e.g., an insulin hormone receptor) relative to a reference standard.

[0076] The amino acid sequence of insulin is strongly conserved throughout evolution, particularly in vertebrates. For example, native canine and porcine insulins differ by only one amino acid from human insulin, native bovine insulin differs by only three amino acids from human insulin, and native feline insulin differs by just four amino acids from human insulin. As used herein, the terms “B-chain or B-chain analog”, “C-peptide” or “C-chain”, and “A-chain or A-chain analog” refer to the peptide segments of an insulin polypeptide as illustrated in FIG. 1. Native insulin is a 51 amino acid hormone containing two peptide chains (i.e., a B-chain and an A-chain) connected via disulfide bonds (e.g., disulfide bonds formed by one or more B-chain cysteine side chain thiols and one or more A-chain cysteine side chain thiols). The A-chain of native insulin is 21 amino acids in length and the B-chain of native insulin is 30 amino acids in length. In the native form of insulin, the A-chain contains one intrachain disulfide bond formed by two A-chain cysteine side chain thiols. For reference purposes, the sequences for the human insulin B-chain of SEQ ID NO: 1 and the human insulin A-chain of SEQ ID NO: 2 are shown below:

(SEQ ID NO: 1)
FVNQHLCGSHLVEALYLVCGERGFFYTPKT

(SEQ ID NO: 2)
GIVEQCCTSI~~CS~~LYQLENYCN.

[0077] As used herein, the term “insulin” or “insulin polypeptide” encompasses mature insulin, proinsulin, proinsulin, and naturally occurring insulin, or analogs thereof. In embodiments, an insulin polypeptide can be a full-length insulin polypeptide or a fragment thereof. In embodiments, modifications to the sequence of the insulin or insulin analog (e.g., amino acid substitutions, deletions, or additions or chemical modifications) may be to the B-chain of insulin, the C-peptide of insulin, the A-chain of insulin, or any combination thereof. In embodiments, the C-chain peptide comprises an amino acid sequence located between the B-chain and the A-chain, and the C-peptide covalently connects the B-chain and A-chain via peptide bonds.

[0078] Insulin is normally constructed as a N-terminus-B-chain:C-chain:A-chain-C-terminus polypeptide, wherein the C-chain is cleaved to make it bioactive. For reference purposes, the sequence of the entire human insulin molecule including the C-chain (i.e., human proinsulin) is shown below with the C-chain in bold:

(SEQ ID NO: 3)
FVNQHLCGSHLVEALYLVCGERGFFYTPKTR**REAEDLQVGGVELGGG**

PGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCN.

[0079] The transformation of the single-chain insulin polypeptide into a bioactive two-chain polypeptide is normally accomplished within the β -cells of the islets of Langerhans prior to glucose-stimulated insulin secretion by two endoproteases, Type I endoproteases, PC1 and PC3, that disrupt the C peptide-B chain connection and PC2, and a Type II endoprotease, that cleaves the C peptide-A chain bond at exactly the right sites. However, cell systems used for the biosynthesis of therapeutic molecules such as insulin (e.g., bacteria, yeast, and mammalian (e.g., HEK and CHO) cell systems) do not possess this pathway, and therefore the transformation must take place after expression and harvesting of the single chain polypeptide using chemical or enzymatic methods. All the known techniques for cleaving the C-chain after expression and harvesting rely on first modifying the C-chain such that it terminates in a lysine just before the N-terminus of the A-chain. Then, using an enzyme selected from the trypsin or Lys-C families, which clips peptide bonds specifically at the C-termini of lysine residues, the single chain-insulin polypeptide is cleaved at the C-terminal lysine of the C-chain and at the C-terminal lysine at the 29th position from the N-terminus of the B-chain. In some cases, the resulting bioactive two-chain insulin is used without reattaching the clipped amino acid at the 30th position from the N-terminus of the B-chain, and in some cases the clipped amino acid at the 30th position from the N-terminus of the B-chain is added back to the molecule using an additional enzymatic method. Such a process works well with insulin because it contains only one lysine in its entire two chain polypeptide form. However, this process cannot be used on the insulin-Fc fusion proteins contained herein, because all known Fc fragments contain multiple lysine residues. The enzymatic cleavage process would, therefore, digest the Fc fragment into non-functional parts,

thereby eliminating the ability of the Fc fragment to prolong the action of the insulin polypeptide in vivo. Therefore, an insulin-Fc fusion protein of the present invention must comprise an insulin polypeptide that does not require C-chain cleavage and is therefore bioactive in its single chain form.

[0080] Several bioactive single chain insulin polypeptides have been described in the art. In all cases, the single chain insulin polypeptides contain C-chains of specific length and composition as well as A-chains and B-chains mutated at specific amino acid sites to achieve electrostatic balance, prevent aggregation, and enhance insulin receptor (IR) binding and/or downstream signaling to achieve bioactivity at levels comparable to that of the native two-chain insulin. Herein, the location of mutations on peptide segments are notated using the name of the segment (e.g., B-chain, C-chain, A-chain) and the number of the amino acid counting from the N-terminus of the segment. For example, the notation “B10” refers to the 10th amino acid from the N-terminus of the amino acid sequence of the B-chain. The notation “A8” refers to the 8th amino acid from the N-terminus of the A-chain. Furthermore, if an amino acid is mutated from its native form to a new amino acid at a particular location, the location is appended with the one letter amino acid code for the new amino acid. For example, B10D refers to an aspartic acid mutation at the 10th amino acid from the N-terminus of the amino acid sequence of the B-chain and A8H refers to a histidine mutation at the 8th amino acid from the N-terminus of the amino acid sequence of the A-chain.

[0081] The insulin polypeptide of an insulin-Fc fusion protein may comprise the amino acid sequence of SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 7.

(SEQ ID NO: 5)
FVNQHLCGSDLVEALALVCGERGFFYTDPTGGGPRRGIVEQCCHSIC
SLYQLENYCN

(SEQ ID NO: 6)
FVNQHLCGSHLVEALYLVCGERGFFYTPKAAAAAAGIVEQCCTSI
CSLYQLENYCN

(SEQ ID NO: 7)
FVNQHLCGSHLVEALYLVCGERGFFYTPKAGGGPRRGIVEQCCTSI
SLYQLENYCN

[0082] In embodiments, the insulin polypeptide of the insulin-Fc fusion protein comprises the amino acid sequence of SEQ ID NO: 4.

(SEQ ID NO: 4)
FVNQHLCGSDLVEALYLVCGERGFFYTDPTGGGPRRGIVEQCCHSIC
SLYQLENYCN

[0083] In embodiments, modifications to the sequence of the insulin or insulin analog (e.g., amino acid substitutions, deletions, or additions or chemical modifications) may be added to either the B-chain of insulin, the C-peptide of insulin, the A-chain of insulin, or any combination thereof. In embodiments, the C-chain peptide comprises an amino acid sequence located between the B-chain and the A-chain, and the C-peptide covalently connects the B-chain and A-chain via peptide bonds.

[0084] In an embodiment, the insulin polypeptide comprises a C-peptide, e.g., a C-chain element which comprises the amino acid sequence GGGPRR (SEQ ID NO: 8).

Fc Fragment

[0085] In embodiments, a fusion protein described herein comprises an Fc fragment, e.g., connected to an insulin polypeptide described herein.

[0086] The term “Fc region,” “Fc domain,” “Fc polypeptide,” or “Fc fragment” as used herein is used to define a C-terminal region of an immunoglobulin heavy chain. The Fc fragment, region, or domain may be a native sequence Fc region or a variant/mutant Fc region. Although the boundaries of the Fc region of an immunoglobulin heavy chain may vary, they generally comprise some or all the hinge region of the heavy chain, the CH2 region of the heavy chain, and the CH3 region of the heavy chain. The hinge region of a feline antibody comprises amino acid sequences that connect the CHI domain of the heavy chain to the CH2 region of the heavy chain and contains one or more cysteines that form one or more interheavy chain disulfide bridges to form a homodimer of the Fc fusion protein from two identical but separate monomers of the Fc fusion protein. The hinge region may comprise all or part of a naturally occurring amino acid sequence or a non-naturally occurring amino acid sequence.

[0087] Each IgG fragment contains a conserved asparagine (N)-glycosylation site in the CH2 domain of each heavy chain of the Fc region. Herein, the notation used to refer to the conserved N-glycosylation site is “cNg”. One way to remove the attached glycan from a synthesized insulin-Fc fusion protein is to mutate the cNg site to prevent the attachment of glycans altogether during production in the host cell. Herein, the notation used to describe a cNg mutation is cNg-(substituted amino acid). For example, if the asparagine at the cNg site is mutated to serine, this mutation is notated as “cNg-S”.

[0088] The absolute position of the cNg site from the N-terminus of the B-chain of the insulin-Fc fusion protein varies depending on the length of the insulin polypeptide, the length of the linker, and any omitted amino acids in the Fc fragment prior to the cNg site. Herein, the notation used to refer to the absolute position of the cNg site in a given insulin-Fc fusion protein sequence (as measured counting from the N-terminus of the B-chain of the insulin-Fc fusion protein) is “NB(number)”. For example, if the cNg site is found at the 138th amino acid position as counted from the N-terminus of the B-chain, the absolute position of this site is referred to as cNg-NB138. As a further example, if the cNg site is found at the 138th amino acid position as counted from the N-terminus of the B-chain, and the asparagine at this site is mutated to serine, this mutation is noted as “cNg-NB138-S”.

[0089] An Fc receptor (FcR) refers to a receptor that binds to an Fc fragment or the Fc region of an antibody. In embodiments, the FcR is a native sequence feline FcR. In embodiments, the FcR is one which binds an Fc fragment or the Fc region of an IgG antibody (a gamma receptor) and includes without limitation, receptors of the Fc(gamma) receptor I, Fc(gamma) receptor IIa, Fc(gamma) receptor IIb, and Fc(gamma) receptor III subclasses, including allelic variants and alternatively spliced forms of these receptors. “FcR” also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus and

is also responsible for the prolonged in vivo elimination half-lives of antibodies and Fc-fusion proteins in vivo. In embodiments, an Fc fragment described herein is capable of binding to mammalian Fc(gamma) or Fc(Rn) receptors, e.g., feline Fc(gamma) or feline Fc(Rn) receptor.

[0090] In embodiments, the Fc fragment comprises the Fc region (e.g., hinge region, CH2 domain, and CH3 domain) of a mammalian IgG, e.g., feline IgG2. In embodiments, the Fc fragment comprises the hinge region (or a fragment thereof) of a feline immunoglobulin (e.g., an IgG, e.g., IgG2). In embodiments, the Fc fragment comprises the Fc region (e.g., CH2 domain and CH3 domain) of feline IgG2.

[0091] Examples of canine Fc fragments are given in SEQ ID NO: 11 (canine IgGA), SEQ ID NO: 12 (canine IgGB), SEQ ID NO: 13 (canine IgGC), and SEQ ID NO: 14 (canine IgGD).

(SEQ ID NO: 11)

RCTDTPPCVPEPLGGPSVLI FPPKPKDILRITRTPVTCVVLDLGRDEPDE
VQISWFVDGKEVHTAKTQPREQPFNGTYRVVSVLPIEHQDWLTKGKFKCRV
NHIDLPSPIERTISKARGRAHKPSVYVLPSPKELSSSDTVSITCLIKDFY
PPDIDVEWQSNQQQEPERKHRMTPPQLDEDGYSFLYSKLSVDKSRWQQGDP
FTCAVMHETLQNHYTDLSSLHSPG

(SEQ ID NO: 12)

DCPKCPAPEMLGGPSVFI FPPKPKDILLIARTPEVTCVVVDLDPEDPEVQI
SWFVDGKMQTAKTQPREQPFNGTYRVVSVLPIGHQDWLKGKQFTCKVNNK
ALPSPPIERTISKARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFPPDI
DVEWQSNQQQEPESKYRTPPQLDEDGYSFLYSKLSVDKSRWQRGDTFICA
VMHEALHNHYTQESLSHSPG

(SEQ ID NO: 13)

CNNCPGCGLLGGPSVFI FPPKPKDILVTARTPTVTCVVVDLDPENPEVQ
ISWFVDSKQVQTANTQPREQSNQTYRVVSVLPIGHQDWLKGKQFKCKVNN
KALPSPPIEBEISKTPGQAHQPNVYVLPSPRDEMKNVTTLTCLVKDFPPPE
IDVEWQSNQQQEPESKYRMTTPQLDEDGYSFLYSKLSVDKSRWQRGDTFIC
AVMHEALHNHYTQISLSHSPG

(SEQ ID NO: 14)

CISPCVPESELGGPSVFI FPPKPKDILRITRTPVTCVVLDLGRDEPDEVQI
SWFVDGKEVHTAKTQPREQPFNSTYRVVSVLPIEHQDWLTKGKFKCRVNI
GLPSPPIERTISKARGQAHQPSVYVLPSPKELSSSDTVTLTCLIKDFPPPE
IDVEWQSNQQQEPESKYHTTAPQLDEDGYSFLYSKLSVDKSRWQQGDTFTC
AVMHEALQNHYTDLSSLHSPG

[0092] In embodiments, the Fc fragment comprises a wild-type feline IgG2 with the following amino acid sequence:

(SEQ ID NO: 21)

GEGPKCPVPEIPGAPSVFI FPPKPKDLSISRTPEVTCVVVDLGPDDSNVQ
ITWFDNTEMHATAKTRPREQPFKSTYRVVSVLPIHLQDWLKGKFKCKVNS
KSLPSAMERTISKAKQPHEPQVYVLPPTQEELSENKVSVTCLIKGFHPDD

- continued

I A V E W E I T G Q P E P E N N Y Q T T P P Q L D S D G T Y F L Y S R L S V D R S H W Q R G N T Y T C
S V S H E A L H S H H T Q K S L T Q S P G .

Linker

[0093] In embodiments, a fusion protein described herein comprises a linker, e.g., between one or more domains of the polypeptide. For example, a fusion protein comprises a linker between the insulin polypeptide and the Fc fragment.

[0094] In embodiments, the linker is a peptide. In embodiments, the peptide linker comprises amino acids (e.g., natural, or unnatural amino acids). In embodiments, the peptide linker can be encoded by a nucleic acid molecule, (e.g., such that a single nucleic acid molecule can encode the various peptides within an insulin polypeptide as well as the peptide linker as well as the Fc fragment). The peptide linker may comprise the amino acid sequence of GGGGAGGGG (SEQ ID NO: 9). In embodiments, the peptide linker comprises the amino acid sequence GGGGSGGG (SEQ ID NO: 10).

Fusion Protein

[0095] Provided herein are fusion proteins, e.g., insulin-Fc fusion proteins. In embodiments, the fusion protein comprises an insulin polypeptide described herein, e.g., in the Insulin polypeptide section herein. In embodiments, the fusion protein comprises an Fc fragment, e.g., an Fc fragment described herein, e.g., in the Fc fragment section herein. In embodiments, the fusion protein comprises a linker between the insulin polypeptide described herein, e.g., in the Insulin polypeptide section herein and the Fc fragment, e.g., an Fc fragment described herein, e.g., in the Fc fragment section herein. Exemplary linkers (e.g., peptide linkers) are described in greater detail in the Linker section herein.

[0096] In embodiments, the insulin polypeptide comprises domains in the following orientation from N- to C-termini: (N-terminus)-B-chain-C-peptide-A-chain-(C-terminus). In embodiments, the insulin polypeptide is located on the N-terminal side of the Fc fragment. In embodiments, the fusion protein comprises domains in the following orientation from N- to C-termini: (N-terminus)-insulin polypeptide-linker-Fc fragment-(C-terminus) (e.g., (N-terminus)-B-chain-C-peptide-A-chain-linker-Fc fragment-(C-terminus)) as illustrated in FIG. 1. In embodiments, the fusion protein, also referred to as the insulin-Fc fusion protein, is comprised of two identical insulin-Fc fusion proteins covalently bound together via one or more disulfide bonds (shown as dotted lines in FIG. 1; total number of disulfide bonds in actuality may be greater or lower than the number shown in FIG. 1). Each insulin-Fc fusion protein comprises an insulin polypeptide molecule containing an insulin B-chain and an insulin A-chain that are connected between the B-chain-C-terminal region and the A-chain-NH₂ terminal region with a C-chain (gray line in FIG. 1), and the A-chain-C-terminal region and Fc-chain amino terminus with a linker, where the insulin-Fc fusion protein sequence terminates in the Fc-CH₃-C-terminal region. Note that the B-chain and A-chain are also linked together via two disulfide bonds (dotted lines in FIG. 1). The A-chain also has an intramolecular disulfide bond (not shown in FIG. 1).

[0097] The full-length sequences of the insulin-Fc fusion proteins of the present technology are provided below:

(SEQ ID NO: 32)
F V N Q H L C G S D L V E A L Y L V C G E R G F F Y T D P T G G G P R R G I V E Q C C H S I C
S L Y Q L E N Y C N G G G G S G G G G E G P K C P V P E I P G A P S V F I F P P K P K D T L
S I S R T P E V T C L V V D L G P D D S N V Q I T W F V D N T E M H T A K T R P R E E Q F N S
T Y R V V S V L P I L H Q D W L K G K E F K C K V N S K S L P S A M E R T I S K A K G Q P H E
P Q V Y V L P P T Q E E L S E N K V S V T C L I K G F H P P D I A V E W E I T G Q P E P E N N
Y Q T T P P Q L D S D G T Y F L Y S R L S V D R S H W Q R G N T Y T C S V S H E A L H S H H T
Q K S L T Q S P G

(SEQ ID NO: 34)
F V N Q H L C G S D L V E A L Y L V C G E R G F F Y T D P T G G G P R R G I V E Q C C H S I C
S L Y Q L E N Y C N G G G G S G G G G E G P K C P V P E I P G A P S V F I F P P K P K D T L
S I S R T P E V T C L V V D L G P D D S N V Q I T W F V D N T E M H T A K T R P R E E Q F S S
T Y R V V S V L P I L H Q D W L K G K E F K C K V N S K S L P S A M E R T I S K A K G Q P H E
P Q V Y V L P P T Q E E L S E N K V S V T C L I K G F H P P D I A V E W E I T G Q P E P E N N
Y Q T T P P Q L D S D G T Y F L Y S R L S V D R S H W Q R G N T Y T C S V S H E A L H S H H T
Q K S L T Q S P G

(SEQ ID NO: 36)
F V N Q H L C G S D L V E A L Y L V C G E R G F F Y T D P T G G G P R R G I V E Q C C H S I C
S L Y Q L E N Y C N G G G G S G G G G E G P K C P V P E I P G A P S V F I F P P K P K D T L
S I S R T P E V T C L V V D L G P D D S N V Q I T W F V D N T E M H T A K T R P R E E Q F K S
T Y R V V S V L P I L H Q D W L K G K E F K C K V N S K S L P S A M E R T I S K A K G Q P H E
P Q V Y V L P P T Q E E L S E N K V S V T C L I K G F H P P D I A V E W E I T G Q P E P E N N
Y Q T T P P Q L D S D G T Y F L Y S R L S V D R S H W Q R G N T Y T C S V S H E A L H S H H T
Q K S L T Q S P G

[0098] The “full aa sequences” of the fusion proteins of SEQ ID NO: 32, SEQ ID NO: 34 and SEQ ID NO: 36 may include a leader sequence. In embodiments, a fusion protein described herein does not include a leader sequence at the N-terminus. In other embodiments, a fusion protein described herein includes a leader sequence, e.g., at the N-terminus. An exemplary leader sequence includes the amino acid sequence MEWSWVFLFSLVTTGVHS (SEQ ID NO: 38). In embodiments, a fusion protein described herein is encoded by a nucleic acid molecule comprising a leader sequence, e.g., for expression (e.g., recombinant expression) in cells (e.g., eukaryotic, e.g., mammalian cells). In embodiments, the leader sequence is part of the fusion protein inside a cell and then the leader sequence is cleaved off, e.g., within the cell or in the cell culture, during expression of the fusion protein into the cell culture media via a process (e.g., an enzymatic process).

[0099] An exemplary nucleic acid sequence encoding a leader sequence includes the nucleic acid sequence ATG-GAATGGAGCTGGGTCTTTCTCTCTCCTG TCAGTAACGACTGGTGTCCACTCC (SEQ ID NO: 39). In other embodiments, a fusion protein described herein is encoded by a nucleic acid molecule not comprising a leader sequence.

[0100] An exemplary nucleic acid sequence encoding the insulin-Fc fusion protein of SEQ ID NO: 32 is given below:

(SEQ ID NO: 32)

ATGGAATGGAGCTGGGTCTTTCTCTTCTCCTGTAGTAACGACTGGTGTC
 CACTCCTTCGTGAACCAGCACCTGTGCGGCTCCGACCTGGTGAAGCTCTG
 TATCTCGTGTGCGGAGCGGGCTTCTTCTACACCGATCCCACTGGAGGC
 GGTCCACGCAGAGGCATCGTGGAACAGTGTGCCACTCCATCTGCTCCCTG
 TACCAGCTGGAAACTACTGCAATGGCGGAGGTGGTTCAGGAGGCGGTGGA
 GGTGAGGGCCCCAAGTGCCCCGTGCCGAGATTCCCGTGCCCCAGCGTG
 TTCATCTTTCCCCAAAACCAAGGACACCCCTGAGCATCAGCAGGACCCCC
 GAGGTGACCTGCCTGGTGGTGGACCTGGGACCCGACGACAGCAACGTGCAG
 ATCACCTGGTTCGTGGACAACACCGAGATGCACACCGCAAGACCAGGCCT
 CGGGAGGAGCAGTTCAACAGCACCTACAGGGTGGTGAGCGTGTGCCCATC
 CTGCACCAGGACTGGCTGAAGGCAAGGAGTTCAAGTGCAAGGTGAACAGC
 AAGAGCCTGCCAGCGCCATGGAGAGGACCATCAGCAAGGCCAAGGGTCAG
 CCCCACGAGCCCCAAGTGTACTGTCTCCCCGACCCAGGAGGAGTTGAGC
 GAGAACAAGTGAGCGTGACCTGCCGTGATCAAGGGCTTCCACCTCCCGAC
 ATCGCCGTGGAGTGGGAGATCACCGCCAGCCTGAGCCGGAAAATAACTAC
 CAGACCACCCCTCCACAGCTGGACAGCGACGGCACCTACTTCCCTTACAGC
 AGGCTGTCTGTGGACCGAAGCATTGGCAAAGGGGCAACACCTACACCTGC
 AGCGTGAGCCACGAGGCTCTGCACAGCCACACCCAGAAGTCTCTGACC
 CAGAGCCCCGGATAG

[0101] An exemplary nucleic acid sequence encoding the insulin-Fc fusion protein of SEQ ID NO: 34 is given below:

(SEQ ID NO: 34)

ATGGAATGGAGCTGGGTCTTTCTCTTCTCCTGTAGTAACGACTGGTGTC
 CACTCCTTCGTGAACCAGCACCTGTGCGGCTCCGACCTGGTGAAGCTCTG
 TATCTCGTGTGCGGAGCGGGCTTCTTCTACACCGATCCCACTGGAGGC
 GGTCCACGCAGAGGCATCGTGGAACAGTGTGCCACTCCATCTGCTCCCTG
 TACCAGCTGGAAACTACTGCAATGGCGGAGGTGGTTCAGGAGGCGGTGGA
 GGTGAGGGCCCCAAGTGCCCCGTGCCGAGATTCCCGTGCCCCAGCGTG
 TTCATCTTTCCCCAAAACCAAGGACACCCCTGAGCATCAGCAGGACCCCC
 GAGGTGACCTGCCTGGTGGTGGACCTGGGACCCGACGACAGCAACGTGCAG
 ATCACCTGGTTCGTGGACAACACCGAGATGCACACCGCAAGACCAGGCCT
 CGGGAGGAGCAGTTTACAGCACCTACAGGGTGGTGAGCGTGTGCCCATC
 CTGCACCAGGACTGGCTGAAGGCAAGGAGTTCAAGTGCAAGGTGAACAGC
 AAGAGCCTGCCAGCGCCATGGAGAGGACCATCAGCAAGGCCAAGGGTCAG
 CCCCACGAGCCCCAAGTGTACTGTCTCCCCGACCCAGGAGGAGTTGAGC
 GAGAACAAGTGAGCGTGACCTGCCGTGATCAAGGGCTTCCACCTCCCGAC
 ATCGCCGTGGAGTGGGAGATCACCGCCAGCCTGAGCCGGAAAATAACTAC

-continued

CAGACCACCCCTCCACAGCTGGACAGCGACGGCACCTACTTCTTTACAGC
 AGGCTGTCTGTGGACCGAAGCATTGGCAAAGGGGCAACACCTACACCTGC
 AGCGTGAGCCACGAGGCTCTGCACAGCCACACCCAGAAGTCTCTGACC
 CAGAGCCCCGGATAG

[0102] An exemplary nucleic acid sequence encoding the insulin-Fc fusion protein of SEQ NO: 36 is given below:

(SEQ ID NO: 36)

ATGGAATGGAGCTGGGTCTTTCTCTTCTCCTGTAGTAACGACTGG
 TGTCACCTCCTTCGTGAACCAGCACCTGTGCGGCTCCGACCTGGTGG
 AAGCTCTGTATCTCGTGTGCGGAGCGGGCTTCTTCTACACCGAT
 CCCACTGGAGGCGGTCCACGACAGGCATCGTGGAACAGTGTGCCA
 CTCCATCTGCTCCCTGTACCAGCTGGAAACTACTGCAATGGCGGAG
 GTGGTTCAGGAGGCGGTGGAGGTGAGGGCCCCAAGTGCCCCGTGCC
 GAGATTCCCGGTGCCCCAGCGTGTTCATCTTTCCCCAAAACCCAA
 GGACACCCCTGAGCATCAGCAGGACCCCCGAGGTGACCTGCCTGGTGG
 TGGACCTGGGACCCGACGACAGCAACGTGCAGATCACCTGGTTCGTG
 GACAACACCGAGATGCACACCGCAAGACCAGGCCTCGGGAGGAGCA
 GTTCAAGAGCACCTACAGGGTGGTGGAGCGTGTGCCCATCTGCACC
 AGGACTGGCTGAAGGGCAAGGAGTTCAAGTGCAAGGTGAACAGCAAG
 AGCCTGCCAGCGCCATGGAGAGGACCATCAGCAAGGCCAAGGGTCA
 GCCCCACGAGCCCCAAGTGTACTGTCTCCCCGACCCAGGAGGAGT
 TGAGCGAGAACAAGTGAGCGTGACCTGCCTGATCAAGGGCTTCCAC
 CCTCCCGACATCGCCGTGGAGTGGGAGATCACCGCCAGCCTGAGCC
 GGAAAATAACTACCAGACACCCCTCCACAGCTGGACAGCGACGGCA
 CCTACTTCTTTACAGCAGGCTGTCTGTGGACCGAAGCCATTGGCAA
 AGGGGCAACACCTACACCTGCAGCGTGGCCACGAGGCTCTGCACAG
 CCACACACCCAGAAGTCTCTGACCCAGAGCCCCGGATAG

[0103] In some embodiments, the fusion protein is in a preparation. In embodiments, the preparation has a percent dimer, e.g., homodimer, of the fusion protein that is greater than about 50%, e.g., greater than about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, 95% or about 100%. In embodiments, the percent dimer, e.g., homodimer, of the fusion protein preparation is 91%, 92%, 91%, 94%, 95%, 96%, 97%, 98%, 99% or 100%. In embodiments, the percent homodimer is about 70% or higher (e.g., 80%, 85%, or 88% or more) and can be made 90% or higher (e.g., 95%, 97%, 98%, 99% or nearly 100%) using one or more processing steps (e.g., ion exchange chromatography, gel filtration, hydrophobic interaction chromatography, etc.). In some embodiments, the % dimer, e.g., homodimer, in the preparation is determined by size-exclusion chromatography which is an analytical separation method that can discriminate between dimers, e.g., homodimers, and higher-order non-covalent Fc fusion protein aggregates (e.g., multimers). In some embodiments, the

% dimer is determined to be greater than 95%, e.g., as determined by size-exclusion chromatography. In some embodiments, the % dimer, e.g., homodimer, is determined to be greater than 99%, e.g., as determined by size-exclusion chromatography. In some embodiments, insulin-Fc fusion proteins with substantially greater homodimer content than other insulin-Fc fusion proteins demonstrate more bioactivity in a subject (e.g., a cat)

Fusion Protein Production

[0104] In embodiments, a fusion protein can be expressed by a vector as described in the Examples section.

Expression and Purification

[0105] In embodiments, a fusion protein can be expressed recombinantly, e.g., in a eukaryotic cell, e.g., mammalian cell or non-mammalian cell. Exemplary mammalian cells used for expression include HEK cells, e.g., HEK293 cells, or CHO cells. In embodiments, cells are transfected with a nucleic acid molecule, e.g., vector, encoding the fusion protein (e.g., where the entire fusion protein is encoded by a single nucleic acid molecule). In other embodiments, cells are transfected with more than one nucleic acid molecule, where each nucleic acid molecule encodes a different domain of the fusion protein. For example, one nucleic acid molecule can encode the insulin polypeptide, and a different nucleic acid molecule can encode the Fc fragment. Cells can be cultured using standard methods in the art.

[0106] In some embodiments, the fusion protein is purified or isolated from the cells (e.g., by lysis of the cells). In other embodiments, the fusion protein is secreted by the cells and, e.g., the fusion protein is purified or isolated from the cell culture media in which the cells were grown. Purification of the fusion protein can include using column chromatography, e.g., affinity chromatography, or using other separation methods that involve size, charge, and/or affinity for certain molecules. In embodiments, purification of the fusion protein involves selecting or enriching for proteins with an Fc fragment, e.g., by using Protein A beads or a Protein A column that cause proteins containing an Fc fragment to become bound with high affinity at neutral solution pH to the Protein A covalently conjugated to the Protein A beads. The bound Fc fusion protein may then be eluted from the Protein A beads by a change in a solution variable (e.g., a decrease in the solution pH). Other separation methods such as ion exchange chromatography and/or gel filtration chromatography can also be employed alternatively or in addition. In embodiments, purification of the fusion protein further comprises filtering or centrifuging the protein preparation. In embodiments, further purification of the fusion protein comprises diafiltration, ultrafiltration, and filtration through porous membranes of various sizes, as well as final formulation with excipients.

[0107] The purified fusion protein can be characterized, e.g., for purity, yield, structure, and/or activity, using a variety of methods, e.g., absorbance at 280 nm (e.g., to determine yield), size exclusion or capillary electrophoresis (e.g., to determine the molecular weight, percent aggregation, and/or purity), mass spectrometry (MS) and/or liquid chromatography (LC-MS) (e.g., to determine purity and/or glycosylation), and/or ELISA (e.g., to determine extent of

binding, e.g., affinity, to an anti-insulin antibody). Exemplary methods of characterization are also described in the Examples section.

Functional Features of Fusion Proteins

[0108] Described herein are methods for treating diabetes (e.g., feline diabetes), the methods comprising the administration of a fusion protein (e.g., a fusion protein described herein) to a subject. In embodiments, a fusion protein described herein is capable of lowering glucose levels (e.g., blood glucose levels) after administration in a subject. In embodiments, the glucose lowering activity of the fusion protein is greater than that of an insulin reference standard. In some embodiments, the duration of activity of the fusion protein can be measured by a decrease, e.g., a statistically significant decrease, in blood glucose relative to a pre-dose glucose level. In embodiments, the fusion protein is long-acting (e.g., has a long half-life, e.g., in serum). In embodiments, the serum half-life of the fusion protein (e.g., in the blood of a subject upon administration) is longer than about 2 hours. In embodiments, the serum half-life of the fusion protein is 4 days, or longer. In embodiments, the serum half-life of the fusion protein is longer than that of an insulin reference standard or control formulation.

[0109] In embodiments, the duration of activity of the fusion protein (e.g., the time during which there is a statistically significant decrease in blood glucose level in a subject relative to a pre-dose level) is longer than about 2 hours. In embodiments, the duration of activity of the fusion protein (e.g., the time during which there is a statistically significant decrease in blood glucose level in a subject relative to a pre-dose level) is longer than about 6 hours, 9 hours, 12 hours, 18 hours, 1 day, 1.5 days, 2 days, 2.5 days, 3 days, 4 days, 5 days or longer. In embodiments, the duration of activity of the fusion protein (e.g., the time during which there is a statistically significant decrease in blood glucose level in a subject relative to a pre-dose level) is longer than that of an insulin reference standard or control formulation.

Methods of Treatment and Characteristics of Subject Selection

[0110] Described herein are methods for interacting with the insulin receptors to lower blood glucose in felines, wherein the method comprises administering to a subject a fusion protein, e.g., a fusion protein described herein. In some embodiments, the subject has been diagnosed with diabetes (e.g., feline diabetes).

[0111] In embodiments, a reference standard used in any method described herein comprises a reference treatment or reference therapy. In some embodiments, the reference comprises a standard of care agent for diabetes treatment (e.g., a standard of care agent for feline diabetes). In some embodiments, the reference standard is a commercially available insulin or insulin analog. In some embodiments, the reference standard comprises a long-lasting insulin, intermediate-lasting insulin, short-lasting insulin, rapid-acting insulin, short-acting, intermediate-acting, long-acting insulin. In some embodiments, the reference standard comprises Vetsulin®, Prozac®, insulin NPH, insulin glargine (Lantus®), or recombinant human insulin.

[0112] In embodiments, a reference standard used in any method described herein includes an outcome, e.g., outcome described herein, of a diabetes therapy (e.g., a feline diabetes therapy).

[0113] In embodiments, a reference standard is a level of a marker (e.g., blood glucose or fructosamine) in the subject prior to initiation of a therapy, e.g., a fusion protein therapy described herein; where the subject has diabetes. In embodiments, the blood glucose level in a cat is greater than 200 mg/dL (e.g., greater than 250 mg/dL, 300 mg/dL, 350 mg/dL, 400 mg/dL or more) prior to initiation of therapy. In embodiments, the fructosamine level in a cat is greater than 250 micromol/L, 350 micromol/L (e.g., greater than 400 micromol/L, 450 micromol/L, 500 micromol/L, 550 micromol/L, 600 micromol/L, 650 micromol/L, 700 micromol/L, 750 micromol/L or more) prior to initiation of therapy. In embodiments, a reference standard is a measure of the presence of or the progression of or the severity of the disease. In embodiments, a reference standard is a measure of the presence of or the severity of the disease symptoms prior to initiation of a therapy, e.g., a fusion protein therapy described herein, e.g., where the subject has diabetes.

Pharmaceutical Compositions and Routes of Administration

[0114] Provided herein are pharmaceutical compositions containing a fusion protein described herein that can be used to lower blood glucose in cats. The amount and concentration of the fusion protein in the pharmaceutical compositions, as well as the quantity of the pharmaceutical composition administered to a subject, can be selected based on clinically relevant factors, such as medically relevant characteristics of the subject (e.g. age, weight, gender, other medical conditions, and the like), the solubility of compounds in the pharmaceutical compositions, the potency and activity of the compounds, and the manner of administration of the pharmaceutical compositions.

[0115] Formulations of the present disclosure include those suitable for parenteral administration. The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by intravenous or subcutaneous injection.

[0116] Examples of suitable aqueous and non-aqueous carriers that may be employed in the pharmaceutical compositions of the disclosure include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, using coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and using surfactants, e.g., Tween-like surfactants. In some embodiments, the pharmaceutical composition (e.g., as described herein) comprises a Tween-like surfactant, e.g., Tween-20 or Tween-80. In some embodiments, the pharmaceutical composition (e.g., as described herein) comprises a Tween-like surfactant, Tween-like surfactant, e.g., polysorbate-20, Tween-20, or Tween-80, at a concentration between about 0.001% and about 2%, or between about 0.005% and about 0.1%, or between about 0.01% and about 0.5%.

[0117] In some embodiments, the fusion protein is administered as a bolus, infusion, or an intravenous push. In some embodiments, the fusion protein is administered through syringe injection, pump, pen, needle, or indwelling catheter.

Methods of introduction may also be provided by rechargeable or biodegradable devices. Various slow release polymeric devices have been developed and tested in vivo in recent years for the controlled delivery of drugs, including proteinaceous biopharmaceuticals. A variety of biocompatible polymers (including hydrogels), including both biodegradable and non-degradable polymers, can be used to form an implant for the sustained release of a compound at a particular target site.

Dosages

[0118] Actual dosage levels of the fusion protein can be varied to obtain an amount of the active ingredient that is effective to achieve the desired therapeutic response for a particular cat. The selected dosage level will depend upon a variety of factors including the activity of the particular fusion protein employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular fusion protein employed, the age, sex, weight, condition, general health and prior medical history of the cat being treated, and like factors well known in the medical arts.

[0119] In general, a suitable dose of a fusion protein will be that amount of the fusion protein that is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. Generally, intravenous, and subcutaneous doses of the fusion protein for a cat will range from about 0.01 to about 10 nanomoles per kilogram of body weight per day, e.g., 0.01-10 nanomoles/kg/day, about 0.1-1 nanomoles/kg/day, about 1-10 nanomoles/kg/day, or about 0.1-10 nanomoles/kg/day. In some embodiments, the fusion protein is administered at a dose greater than or equal to 3 nmol/kg/day (e.g., greater than 4, 5, 6, 10, 20, 30, 40, 50, or 60 nmol/kg/day). In still other embodiments, the fusion protein is administered at a dose between 0.6-60 nanomoles per kilogram of body weight per week, or 0.6-6 nanomoles/kg/week.

[0120] The present disclosure contemplates formulation of the fusion protein in any of the aforementioned pharmaceutical compositions and preparations. Furthermore, the present disclosure contemplates administration via any of the foregoing routes of administration. One of skill in the art can select the appropriate formulation and route of administration based on the condition being treated and the overall health, age, and size of the patient being treated.

EXAMPLES

[0121] The present technology is further illustrated by the following Examples, which should not be construed as limiting in any way.

General Methods, Assays, and Materials

Example 1: Synthesis and Methods of Making an Insulin-Fc Fusion Protein in HEK293 Cells

[0122] Insulin-Fc fusion proteins were synthesized as follows. A gene sequence of interest was constructed using proprietary software (LakePharma, Belmont, Calif.) and was cloned into a high expression mammalian vector. HEK293

cells were seeded in a shake flask 24 hours before transfection and were grown using serum-free chemically defined media. A DNA expression construct that encodes the insulin-Fc fusion protein of interest was transiently transfected into a suspension of HEK293 cells using the (LakePharma, Belmont, Calif.) standard operating procedure for transient transfection. After 20 hours, the cells were counted to determine the viability and viable cell count, and the titer was measured by FortéBio® Octet® (Pall FortéBio LLC, Fremont, Calif.). Additional readings were taken throughout the transient transfection production run. The culture was harvested on or after Day 5.

Example 2: Synthesis and Methods of Making an Insulin-Fc Fusion Protein in HEK293 Cells

[0123] Insulin-Fc fusion proteins are synthesized as follows. A gene sequence of interest is constructed using proprietary software (LakePharma, Belmont, Calif.) and is cloned into a high expression mammalian vector. HEK293 cells are seeded in a shake flask 24 hours before transfection and are grown using serum-free chemically defined media. A DNA expression construct that encodes the insulin-Fc fusion protein of interest is transiently transfected into a suspension of HEK293 cells using the (LakePharma, Belmont, Calif.) standard operating procedure for transient transfection. After 20 hours, the cells are counted to determine the viability and viable cell count, and the titer is measured by FortéBio® Octet® (Pall FortéBio LLC, Fremont, Calif.). Additional readings are taken throughout the transient transfection production run. The culture is harvested on or after Day 5.

Example 3: Purification of an Insulin-Fc Fusion Protein

[0124] Purification of an insulin-Fc fusion protein was performed as follows. Conditioned media supernatants containing the secreted insulin-Fc fusion protein were harvested from the transiently or stably transfected HEK production runs and were clarified by centrifugation. The supernatant containing the desired insulin-Fc fusion protein was run over a Protein A or a Protein G column and eluted using a low pH gradient. Optionally, recovery of the insulin-Fc fusion proteins could be enhanced by reloading of the initial Protein A or Protein G column eluent again onto a second Protein A or Protein G column. Afterwards, the eluted fractions containing the desired protein were pooled and buffer exchanged into 200 mM HEPES, 100 mM NaCl, 50 mM NaOAc, pH 7.0 buffer. A final filtration step was performed using a 0.2 µm membrane filter. The final protein concentration was calculated from the solution optical density at 280 nm. Further optional purification by ion-exchange chromatography (e.g., using an anion exchange bead resin or a cation exchange bead resin), gel filtration chromatography, or other methods was performed, as necessary.

Example 4: Purification of an Insulin-Fc Fusion Protein

[0125] Purification of an insulin-Fc fusion protein is performed as follows. Conditioned media supernatants containing the secreted insulin-Fc fusion protein are harvested from the transiently or stably transfected HEK production runs and are clarified by centrifugation. The supernatant containing the desired insulin-Fc fusion protein is run over a Protein

A or a Protein G column and eluted using a low pH gradient. Optionally, recovery of the insulin-Fc fusion proteins can be enhanced by reloading of the initial Protein A or Protein G column eluent again onto a second Protein A or Protein G column. Afterwards, the eluted fractions containing the desired protein are pooled and buffer exchanged into 200 mM HEPES, 100 mM NaCl, 50 mM NaOAc, pH 7.0 buffer. A final filtration step is performed using a 0.2 µm membrane filter. The final protein concentration is calculated from the solution optical density at 280 nm. Further optional purification by ion-exchange chromatography (e.g., using an anion exchange bead resin or a cation exchange bead resin), gel filtration chromatography, or other methods is performed, as necessary.

Example 5: Structure Confirmation by Non-Reducing and Reducing CE-SDS

[0126] Capillary electrophoresis sodium dodecyl sulfate (CE-SDS) analysis was performed in a LabChip® GXII (Perkin Elmer, Waltham, Mass.) on a solution of a purified insulin-Fc fusion protein dissolved in 200 mM HEPES, 100 mM NaCl, 50 mM NaOAc, pH 7.0 buffer, and the electropherogram was plotted. Under non-reducing conditions, the sample was run against known molecular weight (MW) protein standards, and the eluting peak represented the ‘apparent’ MW of the insulin-Fc fusion protein homodimer. **[0127]** Under reducing conditions (e.g., using beta-mercaptoethanol to break disulfide bonds of the insulin-Fc fusion homodimer), the apparent MW of the resulting insulin-Fc fusion protein monomer is compared against half the molecular weight of the insulin-Fc fusion protein homodimer as a way of determining that the structural purity of the insulin-Fc fusion protein is likely to be correct.

Example 6: Structure Confirmation by Non-Reducing and Reducing CE-SDS

[0128] Capillary electrophoresis sodium dodecyl sulfate (CE-SDS) analysis is performed in a LabChip® GXII (Perkin Elmer, Waltham, Mass.) on a solution of a purified insulin-Fc fusion protein dissolved in 200 mM HEPES, 100 mM NaCl, 50 mM NaOAc, pH 7.0 buffer, and the electropherogram is plotted. Under non-reducing conditions, the sample is run against known molecular weight (MW) protein standards, and the eluting peak represents the ‘apparent’ MW of the insulin-Fc fusion protein homodimer. **[0129]** Under reducing conditions (e.g., using beta-mercaptoethanol to break disulfide bonds of the insulin-Fc fusion homodimer), the apparent MW of the resulting insulin-Fc fusion protein monomer is compared against half the molecular weight of the insulin-Fc fusion protein homodimer as a way of determining that the structural purity of the insulin-Fc fusion protein is likely to be correct.

Example 7: Sequence Identification by LC-MS with Glycan Removal

[0130] To obtain an accurate estimate of the insulin-Fc mass via mass spectroscopy (MS), the sample was first treated to remove naturally occurring glycan that might interfere with the MS analysis. 100 µL of a 2.5 mg/mL insulin-Fc fusion protein dissolved in 200 mM HEPES, 100 mM NaCl, 50 mM NaOAc, pH 7.0 buffer solution was first buffer exchanged into 0.1 M Tris, pH 8.0 buffer containing 5 mM EDTA using a Zeba desalting column (Pierce, Ther-

moFisher Scientific, Waltham, Mass.). 1.67 μ L of PNGase F enzyme (Prozyme N-glycanase) was added to this solution to remove N-linked glycan present in the fusion protein (e.g., glycan linked to the side chain of the asparagine located at the cNg-N site), and the mixture was incubated at 37° C. overnight in an incubator. The sample was then analyzed via LC-MS (NovaBioassays, Woburn, Mass.) resulting in a molecular mass of the molecule which corresponds to the desired homodimer without the glycan. This mass was then further corrected since the enzymatic process used to cleave the glycan from the cNg-asparagine also deaminates the asparagine side chain to form an aspartic acid, and in doing so the enzymatically treated homodimer gains 2 Da overall, corresponding to a mass of 1 Da for each chain present in the homodimer. Therefore, the actual molecular mass is the measured mass minus 2 Da to correct for the enzymatic modification of the insulin-Fc fusion protein structure in the analytical sample.

Example 8: Sequence Identification by LC-MS with Glycan Removal

[0131] To obtain an accurate estimate of the insulin-Fc mass via mass spectroscopy (MS), the sample is first treated to remove naturally occurring glycan that might interfere with the MS analysis. 100 μ L of a 2.5 mg/mL insulin-Fc fusion protein dissolved in 200 mM HEPES, 100 mM NaCl, 50 mM NaOAc, pH 7.0 buffer solution is first buffer exchanged into 0.1 M Tris, pH 8.0 buffer containing 5 mM EDTA using a Zeba desalting column (Pierce, ThermoFisher Scientific, Waltham, Mass.). 1.67 μ L of PNGase F enzyme (Prozyme N-glycanase) is added to this solution to remove N-linked glycan present in the fusion protein (e.g., glycan linked to the side chain of the asparagine located at the cNg-N site), and the mixture is incubated at 37° C. overnight in an incubator. The sample is then analyzed via LC-MS (NovaBioassays, Woburn, Mass.) resulting in a molecular mass of the molecule which corresponds to the desired homodimer without the glycan. This mass is then further corrected since the enzymatic process used to cleave the glycan from the cNg-asparagine also deaminates the asparagine side chain to form an aspartic acid, and in doing so the enzymatically treated homodimer gains 2 Da overall, corresponding to a mass of 1 Da for each chain present in the homodimer. Therefore, the actual molecular mass is the measured mass minus 2 Da to correct for the enzymatic modification of the insulin-Fc fusion protein structure in the analytical sample.

Example 9: % Homodimer by Size-Exclusion Chromatography

[0132] Size-exclusion chromatography (SEC-HPLC) of insulin-Fc fusion proteins was carried out using a Waters 2795HT HPLC (Waters Corporation, Milford, Mass.) connected to a 2998 Photodiode array at a wavelength of 280 nm. 100 μ L or less of a sample containing an insulin-Fc fusion protein of interest was injected into a MAbPac SEC-1, 5 μ m, 4x300 mm column (ThermoFisher Scientific, Waltham, Mass.) operating at a flow rate of 0.2 mL/min and with a mobile phase comprising 50 mM sodium phosphate, 300 mM NaCl, and 0.05% w/v sodium azide, pH 6.2. The MAbPac SEC-1 column operates on the principle of molecular size separation. Therefore, larger soluble insulin-Fc aggregates (e.g., multimers of insulin-Fc fusion protein

homodimers) eluted at earlier retention times, and the non-aggregated homodimers eluted at later retention times. In separating the mixture of homodimers from aggregated multimeric homodimers via analytical SEC-HPLC, the purity of the insulin-Fc fusion protein solution in terms of the percentage of non-aggregated homodimer was ascertained.

Example 10: % Homodimer by Size-Exclusion Chromatography

[0133] Size-exclusion chromatography (SEC-HPLC) of insulin-Fc fusion proteins is carried out using a Waters 2795HT HPLC (Waters Corporation, Milford, Mass.) connected to a 2998 Photodiode array at a wavelength of 280 nm. 100 μ L or less of a sample containing an insulin-Fc fusion protein of interest is injected into a MAbPac SEC-1, 5 μ m, 4x300 mm column (ThermoFisher Scientific, Waltham, Mass.) operating at a flow rate of 0.2 mL/min and with a mobile phase comprising 50 mM sodium phosphate, 300 mM NaCl, and 0.05% w/v sodium azide, pH 6.2. The MAbPac SEC-1 column operates on the principle of molecular size separation. Therefore, larger soluble insulin-Fc aggregates (e.g., multimers of insulin-Fc fusion protein homodimers) elute at earlier retention times, and the non-aggregated homodimers elute at later retention times. In separating the mixture of homodimers from aggregated multimeric homodimers via analytical SEC-HPLC, the purity of the insulin-Fc fusion protein solution in terms of the percentage of non-aggregated homodimer is ascertained.

Example 11: In Vitro IM-9 Insulin Receptor Binding of an Exemplary Insulin-Fc Fusion Protein at 4° C.

[0134] Human IM-9 cells (ATTC# CCL-159) that express human insulin receptor were cultured and maintained in complete RPMI 5% FBS medium at 70-80% confluency. Cultures of IM-9 cells were centrifuged at 250xg (~1000 rpm) for 10 min to pellet the cells. Cells were washed once with HBSS or PBS buffer, resuspended in cold FACS staining medium (HBSS/2 mM EDTA/0.1% Na-azide+4% horse serum) to a concentration of 8x10⁶ cells/mL and kept on ice or 4° C. until test solutions were made. The insulin-Fc protein was diluted in FACS buffer in 1:3 serial dilutions as 2x concentrations in 1.2 mL tubes (approx. 60 μ L volume of each dilution), and the solutions were kept cold on ice until ready for pipetting.

[0135] Biotinylated-RHI was diluted in FACS staining medium to a concentration of 1.25 μ g/mL. 40 μ L of the serially diluted test compound and 8 μ L of 1.25 μ g/mL Biotin-RHI were added into each well of a V bottom microtiter plate, mixed by slow vortexing, and placed on ice. 40 μ L of an IM-9 cell suspension (8x10⁶ cells/mL) was then added to each well by multichannel pipette, mixed again gently and incubated on ice for 30 min to allow competitive binding on the insulin receptor on IM-9 cells. Cells were then washed twice with 275 μ L of ice-cold FACS wash buffer (HBSS/2 mM EDTA/0.1% Na-azide+0.5% horse serum) by centrifuging the V-bottom plate at 3000 rpm for 3 min and aspirating the supernatant. Cells were then resuspended in 404, of FACS staining medium containing 1:100 diluted Streptavidin-PE (Life Technologies) for 20 min on ice. Cells were then washed once with 275 μ L of ice-cold FACS buffer and finally fixed with 3% paraformaldehyde for 10 min at

room temp. Cells were then washed once with 275 μ L of ice-cold FACS buffer and resuspended in 250 μ L of FACS buffer for analysis.

[0136] The V-bottom plates containing cells were then analyzed on a Guava 8-HT flow cytometer (Millipore). Biotinylated-RHI binding to insulin receptor was quantitated by the median fluorescence intensity (MFI) of the cells on the FACS FL-2 channel for each concentration of the test compound. Control wells were labeled only with biotinylated-RHI and were used to calculate the percent (%) inhibition resulting from each test compound concentration. The % inhibition by test compounds of biotinylated-RHI binding on IM-9 cells was plotted against log concentrations of the test compound, and the resulting IC₅₀ values were calculated using GraphPad Prism (GraphPad Software, La Jolla, Calif.) for the test compounds. Lower IC₅₀ values of the test compound therefore indicate greater levels of biotinylated-RHI inhibition at lower concentrations indicating stronger binding of the insulin-Fc fusion protein to the insulin receptor. A control compound, such as unlabeled recombinant human insulin (RHI) was also used as an internal standard to generate an RHI IC₅₀ against which a given compound IC₅₀ could be ratioed (IC₅₀(compound)/IC₅₀(RHI)). Lower IC₅₀ ratios have more similar binding to RHI (stronger binding to insulin receptor), while higher IC₅₀ ratios have weaker binding to the insulin receptor relative to RHI.

Example 12: In Vitro Fc(Gamma) Receptor I Binding Affinity Assay

[0137] The binding of insulin-Fc fusion proteins to the Fc(gamma) receptor I at pH 7.4 was conducted using an ELISA assay as follows. Since canine Fc(gamma) receptor I was not commercially available, human Fc(gamma) receptor I (i.e., rhFc(gamma) receptor I) was used as a surrogate mammalian receptor. Insulin-Fc compounds were diluted to 10 μ g/mL in sodium bicarbonate buffer at pH 9.6 and coated on Maxisorp (Nunc) microtiter plates overnight at 4° C., after which the microplate strips were washed 5 times with PBST (PBS/0.05% Tween-20) buffer and blocked with Superblock blocking reagent (ThermoFisher). Serial dilutions of biotinylated rhFc(gamma) receptor I (recombinant human Fc(gamma)R-I; R&D Systems) were prepared in PBST/10% Superblock buffer from 6000 ng/mL to 8.2 ng/mL and loaded at 100 μ L/well onto the microplate strips coated with insulin-Fc fusion protein. The microtiter plate was incubated for 1 hour at room temperature after which the microplate strips were washed 5 times with PBST and then loaded with 100 μ L/well of streptavidin-HRP diluted 1:10000 in PBST/10% Superblock buffer. After incubating for 45 min, the microplate strips were washed again 5 times with PBST. TMB was added to reveal the bound Fc(gamma) receptor I proteins and stopped with ELISA stop reagent (Boston Bioproducts). The plate was read in an ELISA plate reader at 450 nm, and the OD values (proportional to the binding of rhFc(gamma) receptor I to insulin-Fc protein) were plotted against log concentrations of rhFc(gamma) receptor I added to each well to generate binding curves using GraphPad Prism software.

Example 13: Generalized Procedure for Determination of In Vivo Pharmacodynamics (PD) after Single Administration of Insulin Fc-Fusion Proteins in Dogs

[0138] Insulin-Fc fusion proteins were assessed for their effects on fasting blood glucose levels as follows. N=1, 2, 3

or more healthy, antibody-naïve, dogs weighing approximately 10-15 kg were used, one for each insulin-Fc fusion protein. Dogs were also observed twice daily for signs of anaphylaxis, lethargy, distress, pain, etc., and, optionally for some compounds, treatment was continued for an additional three weekly subcutaneous injections or more to observe if the glucose lowering capability of the compounds lessened over time, a key sign of potential induction of neutralizing anti-drug antibodies. On Day 0, the dogs received a single injection either via intravenous or subcutaneous administration of a pharmaceutical composition containing an insulin Fc-fusion protein homodimer at a concentration between 1 and 10 mg/mL in a solution of between 10-50 mM sodium hydrogen phosphate, 50-150 mM sodium chloride, 0.005-0.05% v/v Tween-80, and optionally a bacteriostat (e.g. phenol, m-cresol, or methylparaben) at a concentration of between 0.02-1.00 mg/mL, at a solution pH of between 7.0-8.0, at a dose of 0.08-0.80 mg insulin-Fc fusion protein/kg (or approximately equivalent to 1.2-12.3 nmol/kg or approximately equivalent to 0.4-4.0 U/kg insulin equivalent on molar basis). On Day 0, blood was collected from a suitable vein immediately prior to injection and at 15, 30, 45, 60, 120, 240, 360, and 480 min and at 1, 2, 3, 4, 5, 6 and 7 days post injection.

[0139] For each time point, a minimum of 1 mL of whole blood was collected. A glucose level reading was immediately determined using a glucose meter (ACCU-CHEK® Aviva Plus), which required approximately one drop of blood. Average % fasting blood glucose levels (% FBGL) from Day 0 to Day 7 were plotted to assess the bioactivity of a given insulin-Fc fusion protein.

Example 14: Generalized Procedure for Determination of In Vivo Pharmacodynamics (PD) after Single Administration of Insulin Fc-Fusion Proteins in Dogs

[0140] Insulin-Fc fusion proteins are assessed for their effects on fasting blood glucose levels as follows. N=1, 2, 3 or more healthy, antibody-naïve, dogs weighing approximately 10-15 kg are used, one for each insulin-Fc fusion protein. Dogs are also observed twice daily for signs of anaphylaxis, lethargy, distress, pain, etc., and, optionally for some compounds, treatment is continued for an additional three weekly subcutaneous injections or more to observe if the glucose lowering capability of the compounds lessened over time, a key sign of potential induction of neutralizing anti-drug antibodies. On Day 0, the dogs receive a single injection either via intravenous or subcutaneous administration of a pharmaceutical composition containing an insulin Fc-fusion protein homodimer at a concentration between 1 and 10 mg/mL in a solution of between 10-50 mM sodium hydrogen phosphate, 50-150 mM sodium chloride, 0.005-0.05% v/v Tween-80, and optionally a bacteriostat (e.g. phenol, m-cresol, or methylparaben) at a concentration of between 0.02-1.00 mg/mL, at a solution pH of between 7.0-8.0, at a dose of 0.08-0.80 mg insulin-Fc fusion protein/kg (or approximately equivalent to 1.2-12.3 nmol/kg or approximately equivalent to 0.4-4.0 U/kg insulin equivalent on molar basis). On Day 0, blood is collected from a suitable vein immediately prior to injection and at 15, 30, 45, 60, 120, 240, 360, and 480 min and at 1, 2, 3, 4, 5, 6 and 7 days post injection.

[0141] For each time point, a minimum of 1 mL of whole blood is collected. A glucose level reading is immediately

determined using a glucose meter (ACCU-CHEK® Aviva Plus), which requires approximately one drop of blood. Average % fasting blood glucose levels (% FBGL) from Day 0 to Day 7 are plotted to assess the bioactivity of a given insulin-Fc fusion protein.

Example 15: Generalized Procedure for
Determination of In Vivo Pharmacodynamics (PD)
after Repeated Administration of Insulin-Fc Fusion
Proteins in Canines

[0142] Insulin-Fc fusion proteins were assessed for their effects on blood glucose levels over repeated injections as follows. Healthy, antibody-naïve, dogs weighing approximately between 5 and 20 kg were used, and each animal was administered doses of an insulin-Fc fusion protein. Animals were observed twice daily for signs of anaphylaxis, lethargy, distress, pain, and other negative side effects, and optionally for some compounds, treatment was continued for up to an additional two to five subcutaneous injections to observe if the glucose lowering capability of the compounds decreased over time, indicating the possible presence of neutralizing anti-drug antibodies in vivo. On Day 0, the animals received a single subcutaneous injection of a pharmaceutical composition containing an insulin Fc-fusion protein in a solution of 10-50 mM sodium hydrogen phosphate, 50-150 mM sodium chloride, 0.005-0.05% v/v Tween-80, and optionally a bacteriostat (e.g. phenol, m-cresol, or methylparaben) at a concentration of between 0.02-1.00 mg/mL, at a solution pH of between 7.0-8.0, at a dose of 0.08-0.80 mg insulin-Fc fusion protein/kg (or approximately equivalent to 1.2-12.3 nmol/kg or approximately equivalent to 0.4-4.0 U/kg insulin equivalent on molar basis). On Day 0, blood was collected from a suitable vein immediately prior to injection and at 15, 30, 45, 60, 120, 240, 360, and 480 min and at 1, 2, 3, 4, 5, 6 and 7 days post injection.

[0143] Subsequent subcutaneous injections were given no more frequently than once-weekly, and in some cases the injections were given at different intervals based on the pharmacodynamics of a given insulin-Fc fusion protein formulation. Subsequent injections for each insulin-Fc fusion protein were adjusted to higher or lower doses, depending on the demonstrated pharmacodynamics of the insulin-Fc fusion protein. For instance, if the dose of a first injection on Day 0 was found to be ineffective at lowering blood glucose, the subsequent dose levels of injected insulin-Fc fusion protein were adjusted upward. In a similar manner, if the dose of a first injection on Day 0 was found to lower glucose in too strong a manner, then subsequent dose levels of injected insulin-Fc fusion protein were adjusted downward. It was also found that interim doses or final doses could be adjusted in a similar manner as needed. For each dose, blood was collected from a suitable vein just immediately prior to injection and at 15, 30, 45, 60, 120, 240, 360, and 480 min and at 1, 2, 3, 4, 5, 6, 7 days (and optionally 14 days) post injection. For each time point, a minimum of 1 mL of whole blood was collected. A glucose level reading was immediately determined using a glucose meter (ACCU-CHEK® Aviva Plus), which required approximately one drop of blood. Average % fasting blood glucose levels (% FBGL) from throughout the study were plotted against time which allows the bioactivity of a fusion protein to be determined.

[0144] To determine the bioactivity of each dose, an area-over-the-curve (AOC) analysis was conducted as fol-

lows. After constructing the % FBGL versus time data, the data was then entered into data analysis software (GraphPad Prism, GraphPad Software, San Diego Calif.). The software was used to first conduct an area-under-the curve analysis (AUC) to integrate the area under the % FBGL vs. time curve for each dose. To convert the AUC data into the desired AOC data, the following equation was used: $AOC = TPA - AUC$; where TPA is the total possible area obtained by multiplying each dose lifetime (e.g., 7 days, 14 days, etc.) by 100% (where 100% represents the $y = 100\%$ of the % FBGL vs. time curve). For example, given a dose lifetime of 7 days and a calculated AUC of 500% FBGL·days, gives the following for AOC: $AOC = (100\% \text{ FBGL} \times 7 \text{ days}) - (500\% \text{ FBGL} \cdot \text{days}) = 200\% \text{ FBGL} \cdot \text{days}$. The analysis can be performed for each injected dose in a series of injected doses to obtain the AOC values for injection 1, injection 2, injection 3, etc.

[0145] As the doses of insulin-Fc fusion protein may vary as previously discussed, it is often more convenient to normalize all calculated AOC values for a given insulin-Fc fusion protein to a particular dose of that insulin-Fc fusion protein. Doing so allows for convenient comparison of the glucose-lowering potency of an insulin-Fc fusion protein across multiple injections, even if the dose levels change across the injections of a given study. Normalized AOC (NAOC) for a given dose is calculated as follows: $NAOC = AOC/D$ with units of % FBGL·days·kg/mg; where D is the actual dose injected into the animal in mg/kg. NAOC values may be calculated for each injection in a series of injections for a given animal and may be averaged across a group of animals receiving the same insulin-Fc fusion protein formulation.

[0146] The NAOC ratio (NAOCR) may also be calculated for each injection in a series of injections for a given animal by taking the NAOC values for each injection (e.g., injections 1, 2, 3 . . . N) and dividing each NAOC for a given injection by the NAOC from injection 1 as follows: $NAOCR = (NAOC(N\text{th injection})/NAOC(\text{injection 1}))$. By evaluating the NAOCR of a given insulin-Fc homodimer fusion protein formulation for the Nth injection in a series of injections, it is possible to determine whether the in vivo glucose lowering activity of a given insulin-Fc fusion protein has substantially retained its bioactivity over a series of N doses (e.g., NAOCR for the Nth dose of greater than 0.5) or whether the in vivo glucose lowering activity of a given insulin-Fc fusion protein has lost a substantial portion of its potency (e.g., NAOCR of the Nth dose is less than 0.5) over a course of N doses, indicating the potential formation of neutralizing anti-drug antibodies in vivo. In preferred embodiments, the ratio of NAOC following the third subcutaneous injection to the NAOC following the first subcutaneous injection is greater than 0.5 (i.e., the NAOCR of the third subcutaneous injection is greater than 0.5).

Example 16: Generalized Procedure for
Determination of In Vivo Pharmacodynamics (PD)
after Repeated Administration of Insulin-Fc Fusion
Proteins in Canines

[0147] Insulin-Fc fusion proteins are assessed for their effects on blood glucose levels over repeated injections as follows. Healthy, antibody-naïve, dogs weighing approximately between 5 and 20 kg are used, and each dog is administered doses of an insulin-Fc fusion protein. Dogs are observed twice daily for signs of anaphylaxis, lethargy,

distress, pain, and other negative side effects, and optionally for some compounds, treatment is continued for up to an additional two to five subcutaneous injections to observe if the glucose lowering capability of the compounds decreases over time, indicating the possible presence of neutralizing anti-drug antibodies *in vivo*. On Day 0, the animals receive a single subcutaneous injection of a pharmaceutical composition containing an insulin Fc-fusion protein in a solution of 10-50 mM sodium hydrogen phosphate, 50-150 mM sodium chloride, 0.005-0.05% v/v Tween-80, and optionally a bacteriostat (e.g. phenol, m-cresol, or methylparaben) at a concentration of between 0.02-1.00 mg/mL, at a solution pH of between 7.0-8.0, at a dose of 0.08-0.80 mg insulin-Fc fusion protein/kg (or approximately equivalent to 1.2-12.3 nmol/kg or approximately equivalent to 0.4-4.0 U/kg insulin equivalent on molar basis). On Day 0, blood is collected from a suitable vein immediately prior to injection and at 15, 30, 45, 60, 120, 240, 360, and 480 min and at 1, 2, 3, 4, 5, 6 and 7 days post injection.

[0148] Subsequent subcutaneous injections are given no more frequently than once-weekly, and in some cases the injections are given at different intervals based on the pharmacodynamics of a given insulin-Fc fusion protein formulation. Subsequent injections for each insulin-Fc fusion protein are adjusted to higher or lower doses, depending on the demonstrated pharmacodynamics of the insulin-Fc fusion protein. For instance, if the dose of a first injection on Day 0 is found to be ineffective at lowering blood glucose, the subsequent dose levels of injected insulin-Fc fusion protein are adjusted upward. In a similar manner, if the dose of a first injection on Day 0 was found to lower glucose in too strong a manner, then subsequent dose levels of injected insulin-Fc fusion protein are adjusted downward. Interim doses or final doses can be adjusted in a similar manner as needed. For each dose, blood is collected from a suitable vein just immediately prior to injection and at 15, 30, 45, 60, 120, 240, 360, and 480 min and at 1, 2, 3, 4, 5, 6, 7 days (and optionally 14 days) post injection. For each time point, a minimum of 1 mL of whole blood is collected. A glucose level reading is immediately determined using a glucose meter (ACCU-CHEK® Aviva Plus), which requires approximately one drop of blood. Average % fasting blood glucose levels (% FBGL) from throughout the study are plotted against time which allows the bioactivity of a fusion protein to be determined.

[0149] To determine the bioactivity of each dose, an area-over-the-curve (AOC) analysis is conducted as follows. After constructing the % FBGL versus time data, the data is then entered into data analysis software (GraphPad Prism, GraphPad Software, San Diego Calif.). The software is used to first conduct an area-under-the curve analysis (AUC) to integrate the area under the % FBGL vs. time curve for each dose. To convert the AUC data into the desired AOC data, the following equation is used: $AOC = TPA - AUC$; where TPA is the total possible area obtained by multiplying each dose lifetime (e.g., 7 days, 14 days, etc.) by 100% (where 100% represents the $y=100\%$ of the % FBGL vs. time curve). For example, given a dose lifetime of 7 days and a calculated AUC of 500% FBGL·days, gives the following for AOC: $AOC = (100\% \text{ FBGL} \times 7 \text{ days}) - (500\% \text{ FBGL} \cdot \text{days}) = 200\% \text{ FBGL} \cdot \text{days}$. The analysis can be performed for each injected dose in a series of injected doses to obtain the AOC values for injection 1, injection 2, injection 3, etc.

[0150] As the doses of insulin-Fc fusion protein may vary as previously discussed, it is often more convenient to normalize all calculated AOC values for a given insulin-Fc fusion protein to a particular dose of that insulin-Fc fusion protein. Doing so allows for convenient comparison of the glucose-lowering potency of an insulin-Fc fusion protein across multiple injections, even if the dose levels change across the injections of a given study. Normalized AOC (NAOC) for a given dose is calculated as follows: $NAOC = AOC/D$ with units of % FBGL·days·kg/mg; where D is the actual dose injected into the animal in mg/kg. NAOC values may be calculated for each injection in a series of injections for a given animal and may be averaged across a group of animals receiving the same insulin-Fc fusion protein formulation.

[0151] The NAOC ratio (NAOCR) may also be calculated for each injection in a series of injections for a given animal by taking the NAOC values for each injection (e.g., injections 1, 2, 3 . . . N) and dividing each NAOC for a given injection by the NAOC from injection 1 as follows: $NAOCR = (NAOC(N\text{th injection})/NAOC(\text{injection 1}))$. By evaluating the NAOCR of a given insulin-Fc homodimer fusion protein formulation for the Nth injection in a series of injections, it is possible to determine whether the *in vivo* glucose lowering activity of a given insulin-Fc fusion protein has substantially retained its bioactivity over a series of N doses (e.g., NAOCR for the Nth dose of greater than 0.5) or whether the *in vivo* glucose lowering activity of a given insulin-Fc fusion protein has lost a substantial portion of its potency (e.g., NAOCR of the Nth dose is less than 0.5) over a course of N doses, indicating the potential formation of neutralizing anti-drug antibodies *in vivo*. In preferred embodiments, the ratio of NAOC following the third subcutaneous injection to the NAOC following the first subcutaneous injection is greater than 0.5 (i.e., the NAOCR of the third subcutaneous injection is greater than 0.5).

Example 17: Assay Protocol for Measuring Anti-Drug Antibodies in Canine Serum

[0152] Maxisorp ELISA Plates (Nunc) are coated with the insulin-Fc fusion protein of interest diluted in coating buffer (pH=9.6 Carbonate-Biocarbonate buffer) at 10 µg/mL overnight at 4° C. for measuring ADAs against the test compound. For measuring ADAs against the insulin portion of the insulin-Fc fusion protein containing an Fc fragment of canine IgG origin, plates are coated with purified insulin at 30 µg/mL in coating buffer. Plates are then washed 5× with PB ST (PBS+0.05% Tween 20) and blocked for at least 1 hour (or overnight) with SuperBlock blocking solution (ThermoFisher, Waltham Mass.). For calculating the ADAs in canine IgG units, strips are directly coated with 1:2 serial dilutions of canine IgG (Jackson ImmunoResearch Laboratories, West Grove Pa.) in pH=9.6 Carb-Biocarb coating buffer at concentrations between 300-4.69 ng/ml overnight at 4° C. and used to create a 7-point pseudo-standard curve. The standards strip plates are also washed and blocked with SuperBlock blocking solution for at least 1 hour (or overnight).

[0153] Test serum samples are diluted to greater than or equal to 1:100 (typically tested as 1:200) in PBST/SB/20% HS sample dilution buffer (PBS+0.1% Tween 20+10% SuperBlock+20% horse serum) and added to the insulin-Fc fusion protein coated (or RHI coated) strips at 100 µL/well in duplicate. Duplicate strips of canine IgG coated standard

strips are also added to each plate and filled with PBST/SB (PBS+0.1% Tween 20+10% SuperBlock) buffer at 100 μ L/well. Plates are incubated for 1 hour at RT and then washed 5 \times with PBST. For detection of ADAs, HRP-conjugated Goat anti-feline IgG F(ab')₂ (anti-feline IgG F(ab')₂ reagent is cross-reacted to canine antibodies; Jackson ImmunoResearch Laboratories, West Grove Pa.), which is diluted in PBST/SB to 1:10000 and added at 100 μ L/well to both sample and standard wells and incubated for 45 minutes at RT in dark. Plates are washed 5 \times with PBST and then one time with deionized water and then developed by adding 100 μ L/well TMB substrate (Invitrogen, ThermoFisher Scientific, Waltham Mass.) for 15-20 minutes at room temperature in the dark. Color development is then stopped by addition of 100 μ L/well of ELISA Stop Solution (Boston Bioproducts) and the absorbance is read at 450 nm using a SpectraMax plate reader within 30 minutes. The anti-drug antibody concentration is determined by interpolating the OD values in the 4-PL pseudo-standard curve using SoftMax Pro Software (Molecular Devices, San Jose Calif.).

[0154] To demonstrate the specificity of the detected ADAs, an "inhibition" assay is carried out. In the drug inhibition ADA assay, serum samples are diluted 1:100 in PBST/SB/20% HS buffer and mixed with an equal volume of 300 μ g/mL of the relevant therapeutic compound (final sample dilution at 1:200 and final inhibitory compound at 150 μ g/mL) and incubated for 30-40 minutes at room temperature to allow anti-drug antibodies to bind the free inhibitor (i.e., the therapeutic compound). After pre-incubation, the samples are added to insulin-Fc fusion protein coated (or RHI coated) strips at 100 μ L/well in duplicate. Samples diluted 1:200 in PBST/SB/20% HS buffer without the inhibitory compound are also tested in the sample plates along with duplicate strips of canine IgG coated standards. Remaining steps of the assay procedure are carried out as described above. The ADAs measured in the drug-inhibited wells are matched with the non-inhibited ADA concentrations to assess the specificity of the ADAs. If significant inhibition of ADA signals is observed in the drug-inhibited wells, this means the ADAs are specific to the therapeutic compound.

Example 18: Assay Procedure for Immunogenic Epitope Identification

[0155] Maxisorp ELISA microplates (Nunc) are coated with a library of insulin-Fc fusion protein homodimer compounds with known amino acid sequences, and the coated plates are blocked in a similar manner as described in the anti-drug antibody ELISA assay Example 17, except that each compound in the library is coated on a separate individual strip of ELISA microplate wells. The compounds in the library comprise a range of insulin-Fc fusion proteins with different insulin polypeptide amino acid compositions, including various B-chain, C-chain, and A-chain amino acid mutations, different linker compositions, and different Fc fragment compositions, including some of human origin. Separately, some plate strip wells are directly coated with 1:2 serial dilutions of canine IgG (Jackson ImmunoResearch Laboratories, West Grove Pa.) for calculating the anti-drug antibodies (ADA) in canine IgG units, respectively, as described in Example 17.

[0156] Serum obtained from individual dogs receiving repeated doses of an insulin-Fc fusion protein is first

screened on the anti-drug antibody ELISA assay (Example 17). Serum samples demonstrating moderate or high positivity (e.g., moderate, or high titers of antibodies) on the assay of Example 17 are serially diluted (1:200 to 1:8000) in PBST/SB/20% HS sample dilution buffer (PBS+0.1% Tween 20+10% SuperBlock+20% horse serum) and added to the plates coated with the library of insulin-Fc fusion protein compounds for 1 hour at RT. Following incubation, the plates are washed 5 times with PBST. For detection of canine antibodies capable of cross-reacting to the coated compound library, HRP conjugated goat anti-feline IgG F(ab')₂ (Jackson ImmunoResearch Laboratories, West Grove Pa.), which is cross-reactive to canine IgGs, is diluted in PBST/SB to 1:10000 and added at 100 μ L/well to both sample and standard wells and incubated for 45 min at RT in the dark. Plates are washed 5 times with PBST, once with deionized water, and developed by the adding 100 μ L/well TMB substrate (Invitrogen, ThermoFisher Scientific, Waltham Mass.) for 15-20 min at RT in the dark. Color development is then stopped by addition of 100 μ L/well of ELISA Stop Solution (Boston Bioproducts, Ashland Mass.) and absorbance is read at 450 nm using a SpectraMax plate reader within 30 min. Anti-compound cross-reactive antibody concentrations present in the serum samples are determined by interpolating the OD values in the 4-PL pseudo-standard curve against the directly coated canine IgG antibody controls using SoftMax Pro Software (Molecular Devices, San Jose Calif.).

[0157] By correlating the resulting antibody concentrations from the assay with the known amino acid compositions of the coated insulin-Fc fusion protein library, one can determine whether particular amino acid mutations or epitopes are responsible for causing none, some, most, or all of the total antibody signal on the assay, indicating no binding, weak binding, or strong binding to various insulin-Fc fusion protein homodimers. The mutations or epitopes responsible for moderate or strong binding are herein referred to as immunogenic "hot spots".

Example 19: Design Process for Obtaining Insulin-Fc Fusion Proteins with High Homodimer Titers and Acceptable Levels of Acute and Repeated Dose Bioactivity in the Target Species

[0158] The process for meeting the design goals described in the Detailed Description of the Invention comprised the following steps. First, the insulin polypeptide of SEQ ID NO: 4 or SEQ ID NO: 5 was combined with a species-specific Fc fragment of a particular IgG isotype and a linker such that the resulting insulin-Fc fusion protein was most likely to yield a long-acting bioactivity product with minimal immunogenicity (e.g., a species-specific IgG isotype was chosen with minimal Fc(gamma)receptor I binding). The DNA sequence coding for the desired fusion protein was prepared, cloned into a vector (LakePharma, San Carlos, Calif.), and the vector was then used to transiently transfect HEK cells according to the procedure described in Example 1. The insulin-Fc fusion protein was then purified according to Examples 3 and the overall protein yield and % homodimer measured according to Example 9. Only candidates with a homodimer titer of greater than 40 mg/L were considered acceptable, because titers less than this level are not likely to result in commercial production titers that meet the stringently low manufacturing cost requirements for veterinary products. Selected insulin-Fc fusion proteins

were then screened for indicators of bioactivity through in vitro insulin receptor binding studies as described in Example 11. Based on experience, only compounds that exhibited IR activity IC50 values less than 5000 nM were deemed likely to exhibit bioactivity in the target species. Although the in vitro IR IC50 value is a useful qualitative screening tool, it utilizes human IM-9 cells which express the human insulin receptor and therefore it may not capture some of the small differences in affinity between the canine IR and the human IR. Furthermore, factors other than insulin receptor binding may influence a compound's bioactivity in vivo (e.g., affinity for canine FcRn to allow for extended pharmacokinetic elimination half-lives in vivo). Therefore, selected insulin-Fc fusion proteins that were acceptable from a manufacturing and IR activity IC50 value standpoint were further screened for bioactivity in the animal of interest (e.g., dog or cat) to screen out any materials with less than the desired potency and/or duration of bioactivity (e.g., NAOC of less than 150% FBGL·days·kg/mg). Again, based on experience, at NAOC values of greater than 150% FBGL·days·kg/mg, the dose requirements in the target species will be sufficiently low to reach an acceptable treatment cost. Lastly, an additional evaluation criterion was added which is mentioned rarely if ever in the art. As discussed in more detail in the Examples below, many insulin-Fc fusion protein embodiments that exhibit acceptable NAOC levels in the target species after the first dose, unexpectedly fail to maintain that level of bioactivity after repeated doses. Furthermore, in most cases the reduction in repeated dose bioactivity in the target species is correlated with the development of neutralizing anti-drug antibodies. This propensity to generate anti-drug antibodies and the failure to maintain activity render such insulin-Fc fusion proteins impractical for use in treating a chronic disease such as canine diabetes. Therefore, only the insulin-Fc fusions proteins exhibiting acceptable levels of repeated dose bioactivity (e.g., NAOCR values greater than 0.50 for the third dose relative to the first dose) with minimal levels of anti-drug antibodies were deemed acceptable for use in the present invention.

Results—Insulin-Fc Fusion Proteins Comprising a Canine Fc Fragment

Example 20: Canine Insulin-Fc Fusion Protein Configurations Comprising the Canine Fc IgGA, IgGC and IgGD Isotypes

[0159] An attempt was made to produce insulin-Fc fusion proteins comprising the insulin polypeptide sequences of SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 7, and SEQ ID NO: 6, and the Fc fragment of the canine IgGA isotype (SEQ ID NO: 11), the canine IgGC isotype (SEQ ID NO: 13) or the canine IgGD isotype (SEQ ID NO: 14) using the peptide linker of SEQ ID NO: 10 or SEQ ID NO: 9. The full amino acid sequences for the resulting insulin-Fc fusion protein configurations are as follows:

(SEQ ID NO: 22)
 FVNQHLGSDLV EALALVCGERGFYDPTGGGPRRGIVEQCCHSICSLYQ
 LENYCNNGGGGAGGGRC TDP P C P V P E P L G G P S V L I F P P K P K D I L R I T R T P
 EVTCVVLDLGR EDPEVQ I S W F V D G K E V H T A K T Q S R E Q Q F N G T Y R V V S V L P I

- continued
 EH Q D W L T G K E F K C R V N H I D L P S P I E R T I S K A R G R A H K P S V Y V L P P S P K E L S
 S S D T V S I T C L I K D F Y P P D I D V E W Q S N G Q Q E P E R K H R M T P P Q L D E D G S Y F L Y
 S K L S V D K S R W Q Q G D P F T C A V M H E T L Q N H Y T D L S L S H S P G
 (SEQ ID NO: 23)
 FVNQHLGSDLV EALYLVCGERGFYDPTGGGPRRGIVEQCCHSICSLYQ
 LENYCNNGGGGSGGGRC TDP P C P V P E P L G G P S V L I F P P K P K D I L R I T R T P
 EVTCVVLDLGR EDPEVQ I S W F V D G K E V H T A K T Q S R E Q Q F N G T Y R V V S V L P I
 EH Q D W L T G K E F K C R V N H I D L P S P I E R T I S K A R G R A H K P S V Y V L P P S P K E L S
 S S D T V S I T C L I K D F Y P P D I D V E W Q S N G Q Q E P E R K H R M T P P Q L D E D G S Y F L Y
 S K L S V D K S R W Q Q G D P F T C A V M H E T L Q N H Y T D L S L S H S P G
 (SEQ ID NO: 24)
 FVNQHLGSHLVEALYLVCGERGFYTPKAGGPRRGIVEQCCTSICSLYQ
 LENYCNNGGGGSGGGRC TDP P C P V P E P L G G P S V L I F P P K P K D I L R I T R T P
 EVTCVVLDLGR EDPEVQ I S W F V D G K E V H T A K T Q S R E Q Q F N G T Y R V V S V L P I
 EH Q D W L T G K E F K C R V N H I D L P S P I E R T I S K A R G R A H K P S V Y V L P P S P K E L S
 S S D T V S I T C L I K D F Y P P D I D V E W Q S N G Q Q E P E R K H R M T P P Q L D E D G S Y F L Y
 S K L S V D K S R W Q Q G D P F T C A V M H E T L Q N H Y T D L S L S H S P G
 (SEQ ID NO: 25)
 FVNQHLGSHLVEALYLVCGERGFYTPKAAAAAAK GIVEQCCTSICSLY
 QLENYCNNGGGGSGGGRC TDP P C P V P E P L G G P S V L I F P P K P K D I L R I T R T
 PEVTCVVLDLGR EDPEVQ I S W F V D G K E V H T A K T Q S R E Q Q F N G T Y R V V S V L P
 IEH Q D W L T G K E F K C R V N H I D L P S P I E R T I S K A R G R A H K P S V Y V L P P S P K E L
 S S S D T V S I T C L I K D F Y P P D I D V E W Q S N G Q Q E P E R K H R M T P P Q L D E D G S Y F L
 Y S K L S V D K S R W Q Q G D P F T C A V M H E T L Q N H Y T D L S L S H S P G

[0160] FIG. 3 illustrates a side-by-side sequence comparison of SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24 and SEQ ID NO: 25. “*” represents complete homology across all sequences at a given sequence position, while “:”, “.” or spaces refer to conservative, moderate, or very different amino acid mutations across the sequences at a given sequence position, respectively.

[0161] The insulin-Fc fusion protein configuration of SEQ ID NO: 27, comprising the insulin polypeptide of SEQ ID NO: 5, the peptide linker of SEQ ID NO: 9 and the canine IgGC Fc fragment of SEQ ID NO: 13 yielded no material at all. The insulin-Fc fusion protein configuration of SEQ ID NO: 28, comprising the insulin polypeptide of SEQ ID NO: 5, the peptide linker of SEQ ID NO: 9 and the canine IgGD Fc fragment of SEQ ID NO: 14 yielded a good protein yield of 134 mg/L, however the material was highly aggregated with a % homodimer of 12.3% and a homodimer titer of 16.5 mg/L, which is significantly less than the required homodimer titer of 40 mg/L.

(SEQ ID NO: 27)
 FVNQHLGSDLV EALALVCGERGFYDPTGGGPRRGIVEQCCHSICSLYQ
 LENYCNNGGGGAGGGC N N C P C P G C G L L G G P S V F I F P P K P K D I L V T A R T P T V
 T C V V D L D P E N P E V Q I S W F V D S K Q V Q T A N T Q P R E E Q S N G T Y R V V S V L P I G H

-continued

QDWLSGKQFKCKVNNKALPSPIEEII SKTPGQAHQPNVYVLPSPSRDEMSKN
 TVTLTCLVKDFFPPEIDVEWQSNQQEPEPEPKYRMTTPQLDEDEGSYFLYSKL
 SVDKSRWQRGDTFICAVMHEALHNHYTQISLSHSPG
 (SEQ ID NO: 28)
 FVNQHLGSDLVLEALALVCGERGFYTDPTGGGPRRGIVEQCCHSICSLYQ
 LENYCNNGGGGAGGGGCI SPCVPPELGGPSVFI FPPKPKDILRITRTPET
 CVVLDLGPEDPEVQISWVFDGKQMTAKTQPREQQFNSTYRVVSVLPIEHQ
 DWLTGKEFKCRVNHIGLPSPIERTISKARGQAHQPSVYVLPSPKELSSSD
 TVTLTCLIKDFFPPEIDVEWQSNQQEPEPEPKYHTTAPQLDEDEGSYFLYSKL
 SVDKSRWQQGDTFTCAVMHEALQNHYTDL SLSHSPG

(SEQ ID NO: 26)
 FVNQHLGSDLVLEALALVCGERGFYTDPTGGGPRRGIVEQCCHSICSLYQ
 LENYCNNGGGGAGGGGDCPKCPAPEMLGGPSVFI FPPKPKDILLIARTPEVT
 CVVVDLDPEDPEVQISWVFDGKQMTAKTQPREQQFNSTYRVVSVLPIGHQ
 DWLKGKQFTCKVNNKALPSPERTISKARGQAHQPSVYVLPSPREELSKNT
 VSLTCLIKDFFPPPIDVVEWQSNQQEPEPEPKYRTTTPQLDEDEGSYFLYSKLS
 VDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG

Example 22: In Vitro IM-9 Insulin Receptor Binding of an Exemplary Insulin-Fc Fusion Protein at 4° C.

[0162] The insulin-Fc fusion protein configurations of SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 27 and SEQ ID NO: 28 were synthesized in HEK cells according to Example 1 and purified according to Example 3. The structure of the insulin-Fc fusion protein was confirmed according to Example 5 by non-reducing and reducing CE-SDS, and the sequence was further identified by LC-MS with glycan removal according to Example 7. The % homodimer was measured by size-exclusion chromatography according to Example 9. The results are given in Table 1. In summary, despite mutating the insulin polypeptide and/or the linker, there was no embodiment based on the canine IgGA Fc fragment (SEQ ID NO: 11) with a low enough degree of aggregation and a high enough titer of the desired homodimer.

[0164] In vitro insulin receptor binding for the insulin-Fc fusion protein configurations of SEQ ID NO: 22 (IgGA), SEQ ID NO: 26 (IgGB), SEQ ID NO: 27 (IgGC) and SEQ ID NO: 28 (IgGD) were tested according to the procedure of Example 11. As shown in Table 2, the insulin-Fc fusion protein of SEQ ID NO: 22 demonstrated in IR binding IC50 of 2733 and the insulin-Fc fusion protein of SEQ ID NO: 28 demonstrated an IR binding IC50 of greater than 5000 nM, indicating that neither insulin-Fc fusion protein configuration was highly unlikely to show bioactivity in vivo. The insulin-Fc fusion protein of SEQ ID NO: 27 had no yield and so the IR binding IC50 was not measure. However, the insulin-Fc fusion protein of SEQ ID NO: 26 demonstrated an IC50 of 28 nM indicating that this sequence was likely to be bioactive in vivo.

TABLE 1

Homodimer Titers for Insulin-Fc Fusion Protein Configurations Utilizing Canine IgG Fc Fragments				
SEQ ID NO:	Canine IgG	Protein Yield (mg/L)	% Homodimer	Homodimer Titer (mg/L)
SEQ ID NO: 22	IgGA	22	24%	5.28
SEQ ID NO: 23	IgGA	21	19%	3.99
SEQ ID NO: 24	IgGA	11	Not tested	≤11
SEQ ID NO: 25	IgGA	18	Not tested	≤18
SEQ ID NO: 27	IgGC	0	Not tested	0
SEQ ID NO: 28	IgGD	134	12%	16.5

Example 21: Canine Insulin-Fc Fusion Protein Configurations Comprising the Canine Fc IgGB Isotype

[0163] On the other hand, replacing the canine IgGA Fc fragment (SEQ ID NO: 11) with the canine IgGB Fc fragment (SEQ ID NO: 12) yielded a much less aggregated compound with a comparatively high titer of the desired homodimer. The insulin-Fc fusion protein configuration of SEQ ID NO: 26, comprising the insulin polypeptide of SEQ ID NO: 5, the linker of SEQ ID NO: 9 and the canine IgGB Fc fragment of SEQ ID NO: 12 had a protein yield of 80 mg/L and a % homodimer of 93% resulting in a homodimer titer of 74.7 mg/L, which surpassed the required homodimer titer of 40 mg/L.

TABLE 2

Homodimer Titers for Sequences Utilizing Native Canine IgGA, IgGB, IgGC, and IgGD Fc Fragments					
SEQ ID NO:	IgG Fragment	Protein Yield (mg/L)	% Homodimer	Homodimer Titer (mg/L)	IR Binding, IC50 (nM)
SEQ ID NO: 22	IgGA	22	24%	5.28	2,733
SEQ ID NO: 26	IgGB	80	93%	74.4	28
SEQ ID NO: 27	IgGC	0	Not tested	0	DNM*
SEQ ID NO: 28	IgGD	134	12%	16.5	>5000

*DNM = Did Not Measure

Example 23: In Vivo Efficacy of an Insulin-Fc Fusion Protein Comprising the Insulin Polypeptide of SEQ ID NO: 5 with a Canine IgGB Isotype Fc Fragment

[0165] Given the promising homodimer titer and insulin receptor activity results in Examples 21 and 22, the insulin-Fc fusion protein of SEQ ID NO: 26 was tested for in vivo bioactivity according to Example 13 following an intravenous injection in each of N=3 healthy, antibody-naïve, beagle dogs weighing approximately 10 kg. In a separate experiment, the compound was administered subcutaneously to N=3 naïve beagle dogs. FIG. 5 shows the % FBGL versus time for a single intravenous administration of the insulin-Fc fusion protein of SEQ ID NO: 26, and FIG. 6 shows the % FBGL vs. time for a single subcutaneous administration of the insulin-Fc fusion protein of SEQ ID

NO: 26, both of which demonstrate that the insulin-Fc fusion protein of SEQ ID NO: 26 is significantly bioactive in dogs.

[0166] The NAOC was calculated according to the procedure of Example 15 to determine the relative bioactivity and duration of action of the insulin-Fc fusion protein. The NAOC of the insulin-Fc fusion protein of SEQ ID NO: 26 injected intravenously was 399% FBGL·days·kg/mg which was 3.8 times the NAOC of the insulin-Fc fusion protein of SEQ ID NO: 22 injected intravenously, illustrating significantly increased bioactivity for the insulin-Fc fusion protein comprising the canine IgGB Fc fragment versus the insulin-Fc fusion protein comprising the canine IgGA Fc fragment. The in vivo bioactivity of SEQ ID NO: 22 is illustrated in FIG. 2. The NAOC of the insulin-Fc fusion protein of SEQ ID NO: 26 injected subcutaneously was 366% FBGL·days·kg/mg, demonstrating a level of bioactivity via subcutaneous administration that is like that obtained via intravenous administration. The in vivo bioactivity of SEQ ID NO: 26 across two doses is illustrated in FIG. 8.

Example 24: In Vivo Immunogenicity Screening after Repeated Subcutaneous Doses of the Insulin-Fc Fusion Protein Comprising the Insulin Polypeptide of SEQ ID NO: 26 with a Canine IgGB Isotype Fc Fragment

[0167] Next, the repeated dose subcutaneous bioactivity of the insulin-Fc fusion protein of SEQ ID NO: 26 was tested in dogs as per the method described in Example 15. N=3 animals were dosed subcutaneously at Day 0, at Day 35, and at Day 42, and the % FBGL was measured for the 7-day window after each dose according to Example 15. The NAOC and NAOCR were calculated according to the procedure of Example 15 for each repeated subcutaneous injection. As illustrated in Table 3, repeated subcutaneous dosing in dogs unexpectedly revealed a significant decay in bioactivity by the third dose as measured by a significant decrease in the NAOCR (i.e., the NAOCR for the third injection was only 0.40, or 40%, of the NAOC for the first injection).

TABLE 3

NAOC per Dose and NAOCR for Repeated Doses of SEQ ID NO: 26		
Injection Number of SEQ ID NO: 26	NAOC (% FBGL · days · kg/mg)	NAOCR (ratioed to Week 1)
1	330	1.0
2	339	1.1
3	115	0.4

[0168] Without being bound to any particular explanation, it was postulated that the cause of the significant reduction in bioactivity of the insulin-Fc fusion protein of SEQ ID NO: 26 after the third repeated subcutaneous dose in dogs was due to the development of anti-drug antibodies that neutralized its biological activity. Anti-drug antibodies may be directed against the insulin polypeptide, linker, or Fc-fragment portions of an insulin-Fc fusion protein. The immunogenic response manifests as interactions between antigen presenting cells, T-helper cells, B-cells, and their associated cytokines, which may lead to the production of endogenous antibodies against the drug (e.g., anti-drug antibodies). Binding antibodies are all isotypes capable of binding the

insulin-Fc fusion protein, and these may be detected in an immunoassay as described in Example 17. Neutralizing antibodies that inhibit functional activity of the insulin-Fc fusion protein are generally directed against an epitope that is required for bioactivity. To assess whether this was the case, serum that was collected prior to the administration of each dose and at the end of the experiment described in Examples 11 was tested to quantify the levels of anti-drug antibodies according to Example 17. As shown in FIG. 7, levels of anti-drug antibodies did indeed increase with multiple subcutaneous administrations of the compound, indicating that the generation of neutralizing anti-drug antibodies were the likely cause for the reduction in the NAOCR after the third injection of the insulin Fc-fusion protein of SEQ ID NO: 26.

Example 25: Non-Glycosylated Insulin-Fc Fusion Protein Comprising the Insulin Polypeptide of SEQ ID NO: 5 with Canine IgGB Isotype Fc Fragments to Reduce the Potential Risk of Immunogenicity

[0169] As shown in Example 21, Example 22 and Example 23, the insulin-Fc fusion protein of SEQ ID NO: 26 showed acceptable % homodimer content, homodimer titer, and bioactivity in dogs; however, its use for a chronic disease such as diabetes is compromised by the reduction in bioactivity and generation of anti-drug antibodies (Example 24) with repeated subcutaneous dosing. Without being bound to any particular theory, one possible cause of the generation of anti-drug antibodies and the reduction in bioactivity is the increased interaction of the canine IgGB Fc fragment with various receptors of the canine immune system (e.g., Fc(gamma) receptors, e.g., Fc(gamma)RI). Nevertheless, the canine IgGB isotype was the only one of the four canine IgG isotypes that, when used for the Fc fragment, resulted in an insulin-Fc fusion protein meeting the manufacturability and single-dose bioactivity design goals (Example 19). As described in the Detailed Description of the Invention, one method for reducing the Fc(gamma) interaction involves mutating the Fc fragment cNg site to prevent glycosylation during synthesis in the host cell.

[0170] Therefore, cNg site mutations were made to the Fc fragment region of SEQ ID NO: 26 to reduce the binding affinity of the Fc fragment for Fc(gamma) receptors in vivo, as measured by binding in an in vitro human Fc(gamma)RI assay described in Example 12. Verification of the lack of glycan were performed using the LC-MS method of Example 7, but with omission of the PNGase F treatment step. The position of the cNg site in the insulin-Fc fusion protein of SEQ ID NO: 26 is cNg-NB139. Mutations to SEQ ID NO: 26 included SEQ ID NO: 29 comprising a mutation of cNg-NB139-S, SEQ ID NO: 30 comprising a mutation of cNg-NB139-Q together with reverting to the native B10H and A8T, and SEQ ID NO: 31 comprising removal of the aspartic acid at the N-terminus of the Fc fragment (des hinge D) a mutation of cNg-NB138-K (due to the des hinge D mutation, the cNg site for SEQ ID NO: 31 is NB138 instead of NB139). The full amino acid sequences of the cNg-mutated insulin-Fc fusion proteins are listed below (with the cNg position in bold).

(SEQ ID NO: 29)
 FVNQHLGSDLVEALALVCGERGFYTDPTGGGPRRGIVEQCCHSICSLYQ
 LENYCNNGGGGAGGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVT
 CVVVDLDPEDPEVQISWVFDGKQMKTAKTQPREEQFSGTYRVVSVLPIGHQ
 DWLKGKQFTCKVNNKALPSPERTISKARGQAHQPSVYVLPSPREELSKNT
 VSLTCLIKDFFPPDIDVEWQSNQQEPEESKYRTTPQLDEDEGSYFLYSKLS
 VDKSRWQRGDTFICAVMHHEALHNHYTQESLSHSPG

(SEQ ID NO: 30)
 FVNQHLGSHLVEALALVCGERGFYTDPTGGGPRRGIVEQCCTSICSLYQ
 LENYCNNGGGGAGGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVT
 CVVVDLDPEDPEVQISWVFDGKQMKTAKTQPREEQFQGTYRVVSVLPIGHQ
 DWLKGKQFTCKVNNKALPSPERTISKARGQAHQPSVYVLPSPREELSKNT
 VSLTCLIKDFFPPDIDVEWQSNQQEPEESKYRTTPQLDEDEGSYFLYSKLS
 VDKSRWQRGDTFICAVMHHEALHNHYTQESLSHSPG

(SEQ ID NO: 31)
 FVNQHLGSDLVEALALVCGERGFYTDPTGGGPRRGIVEQCCHSICSLYQ
 LENYCNNGGGGAGGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVTC
 VVVDLDPEDPEVQISWVFDGKQMKTAKTQPREEQFKGTYRVVSVLPIGHQD
 WLKGKQFTCKVNNKALPSPERTISKARGQAHQPSVYVLPSPREELSKNTV
 SLTCLIKDFFPPDIDVEWQSNQQEPEESKYRTTPQLDEDEGSYFLYSKLSV
 DKSRWQRGDTFICAVMHHEALHNHYTQESLSHSPG

[0171] The insulin-Fc fusion proteins were manufactured in HEK293 cells according to Example 1 and purified using a Protein A column according to Example 3. The structures of the insulin-Fc fusion proteins were confirmed according to Example 5 by non-reducing and reducing CE-SDS, and the sequences were further identified by LC-MS with glycan removal according to Example 7. The % homodimer was measured by size-exclusion chromatography according to Example 9. As shown in Table 4, the homodimer titers of the insulin-Fc fusion proteins of SEQ ID NO: 29, SEQ ID NO: 30 and SEQ ID NO: 31 met the design goal for homodimer titer.

Example 26: Screening of Canine Serum
 Containing Anti-Drug Antibodies and Identification
 of Potential Immunogenic Epitopes at the B10D
 and A8H Positions of the Insulin Polypeptide

[0172] It was hypothesized, therefore, that the insulin polypeptide of SEQ ID NO: 5 may unexpectedly contain specific epitopes (i.e., immunogenic “hot spots”) against which the dog’s immune system is directed. Therefore, the binding specificity of the antibodies present in the serum samples described in Example 17 were evaluated according to the general procedure of Example 18. The analysis of the antibody-containing serum samples from the repeated dosing of the insulin-Fc fusion protein of SEQ ID NO: 26 (Example 24) against the coated insulin-Fc fusion protein library demonstrated that there were unexpectedly two primary “hot spots” present within the insulin polypeptide sequence of SEQ ID NO: 5: the aspartic acid mutation at the 10th position from the N-terminus of the B-chain (i.e., B10), and, separately, the histidine mutation at the 8th position

from the N-terminal end of the A-chain (i.e., A8). The results suggest that insulin-Fc fusion proteins comprising insulin polypeptide amino acid compositions containing these two particular amino acid mutations are likely to be immunogenic in dogs and therefore likely to give rise to anti-drug antibodies that neutralize the bioactivity after repeated injections. The insulin-Fc fusion protein configurations of SEQ ID NO: 29 and SEQ ID NO: 31 however met the design requirements for IR binding IC50, while the insulin-Fc fusion protein configuration of SEQ ID NO: 30 (where the B10D and A8H mutations were restored to the native B10H and A8T) demonstrated an IR binding IC50 of >5000 nM, indicating that this insulin-Fc fusion protein configuration was highly unlikely to be bioactive in vivo. This indicated that the B10H and A8T mutations in these insulin-Fc fusion protein configurations appeared to be necessary to achieve in vivo bioactivity.

TABLE 4

Homodimer Titers and IR Binding for Insulin-Fc Fusion Protein Configurations Utilizing Canine IgGB Fc with cNg Mutations					
SEQ ID NO:	cNg MUTA-TION	Protein Yield (mg/L)	% Homo-dimer	Homo-dimer Titer (mg/L)	IR Binding _{IC50} (nM)
SEQ ID NO: 29	S	76.7	98.3%	75.4	145
SEQ ID NO: 30	Q	21.4	98.7%	21.1	>5000
SEQ ID NO: 31	K	45.4	98.1%	44.5	147

Example 27: Evaluation of In vivo Bioactivity and Immunogenicity of Non-Glycosylated Insulin-Fc Fusion Protein Configurations of with cNg Mutations of the Canine IgGB Isotype Fc Fragment

[0173] To determine if the insulin-Fc fusion protein of SEQ ID NO: 29, containing the cNg-S mutation, improved the repeated dose bioactivity performance in dogs, the compound was administered subcutaneously to N=1 dog on Day 0, Day 14, Day 21 and on Day 28 according to the procedure of Example 15. The % FBGL across these doses for SEQ ID NO: 29 is illustrated in FIG. 9. When the dog’s % FBGL dropped too low, the dog was given food to raise the blood glucose to a safe level. The NAOC for the first injection measured over the first 7 days following the injection was 403% FBGL-days/kg/mg, showing that the insulin-Fc fusion protein of SEQ ID NO: 29 was satisfactorily bioactive in vivo. The NAOC and NAOCR were also measured for each subsequent dose according to the general procedure of Example 15, calculated from the time the dose was administered until just before the next dose was administered. The NAOC and the NAOCR shown in Table 5 illustrate that the insulin-Fc fusion protein of SEQ ID NO: 29 exhibited an NAOCR that decreased significantly on doses 2 and 3 of a three dose regimen. Therefore, the insulin-Fc fusion protein of SEQ ID NO: 29, containing the cNg-S mutation, was unable to demonstrate repeated dose bioactivity in dogs.

TABLE 5

NAOC per Dose for Repeated Doses of SEQ ID NO: 29		
Injection Number of SEQ ID NO: 29	NAOC (% FBGL · days · kg/mg)	NAOCR
1	403	1.0
2	508	0.3
3	128	0.32

[0174] To determine if the insulin-Fc fusion protein of SEQ ID NO: 30, containing the cNg-Q mutation, improved the repeated dose bioactivity performance in dogs, the compound was administered subcutaneously to N=1 dog on Day 0 and on Day 28 according to the procedure of Example 15. The % FBGL across these doses for SEQ ID NO: 30 is illustrated in FIG. 10. When the dog's % FBGL dropped too low, the dog was given food to raise the blood glucose to a safe level. The NAOC for the first injection measured over the first 7 days following the injection was 24.2% FBGL·days·kg/mg, showing that the insulin-Fc fusion protein of SEQ ID NO: 30 did not show satisfactory bioactive in vivo. The NAOC and NAOCR were also measured for the subsequent dose according to the general procedure of Example 15, calculated from the time the dose was administered until just before the next dose was administered. The NAOC and the NAOCR shown in Table 6 illustrate that the insulin-Fc fusion protein of SEQ ID NO: 30 exhibited 0 NAOC and NAOCR for the second dose. Therefore, the insulin-Fc fusion protein of SEQ ID NO: 30, containing the cNg-Q mutation, was unable to demonstrate repeated dose bioactivity in dogs.

TABLE 6

NAOC per Dose for Repeated Doses of SEQ ID NO: 30		
Injection Number of SEQ ID NO: 30	NAOC (% FBGL · days · kg/mg)	NAOCR
1	403	1.0
2	0	0.0

[0175] To determine if the insulin-Fc fusion protein of SEQ ID NO: 31, containing the cNg-K mutation together with the des hinge D omission (the Fc fragment of SEQ ID NO: 16) improved the repeated dose bioactivity performance in dogs, the compound was administered subcutaneously to N=1 dog on Day 0, Day 7, and Day 14 according to the procedure of Example 15. The % FBGL across these doses for SEQ ID NO: 31 is illustrated in FIG. 11. When the dog's % FBGL dropped too low, the dog was given food to raise the blood glucose to a safe level. The NAOC for the first injection was 178% FBGL·days·kg/mg, showing that the insulin-Fc fusion protein of SEQ ID NO: 31 was satisfactorily bioactive in vivo. The NAOC and NAOCR were also measured for each subsequent dose according to the general procedure of Example 15, calculated from the time the dose was administered until just before the next dose was administered. The NAOC and the NAOCR shown in Table 7 illustrate that the insulin-Fc fusion protein configuration of SEQ ID NO: 31 maintains an NAOCR greater than 0.6 throughout the three doses. Therefore, unexpectedly, the insulin-Fc fusion protein of SEQ ID NO: 31, containing the cNg-K mutation with des hinge D, was the only non-glycosylated mutant of the insulin-Fc fusion protein

of SEQ ID NO: 26 resulting in significantly improved repeated dose bioactivity in dogs.

TABLE 7

NAOC per Dose for Repeated Doses of SEQ ID NO: 31		
Injection Number of SEQ ID NO: 31	NAOC (% FBGL · days · kg/mg)	NAOCR
1	178	1.0
2	382	2.2
3	238	1.3

Example 28: In Vivo Pharmacodynamics (PD) after a Single Administration of an Insulin Fc-Fusion Protein in Felines

[0176] A bioactive insulin-Fc fusion homodimer construct of SEQ ID NO: 32 was synthesized according to Example 1 and was assessed for its effects on fasting blood glucose levels as follows. N=3 healthy, antibody-naïve, cats weighing approximately 5 kg were used. On Day 0, the cats received a single injection of a pharmaceutical composition containing an insulin Fc-fusion protein homodimer of SEQ ID NO: 32 in a solution of 50 mM sodium hydrogen phosphate, 150 mM sodium chloride, and 0.02% v/v Tween-80 at pH 7.5, at a dose of 6 nmol/kg (equivalent to 0.39 mg Fc fusion protein/kg or 1.9 U/kg insulin equivalent on molar basis). On Day 0 immediately prior to injection and at 15, 30, 45, 60, 120, 240, 360, and 480 minutes and at 1, 2, 3, 4, 5, 6, 7, and 8 days post injection blood was collected from a suitable vein.

[0177] For each time point, a minimum of 1 mL of whole blood was collected. A glucose level reading was immediately determined using a glucose meter (ACCU-CHEK® Aviva Plus), which requires approximately one drop of blood. Average % fasting blood glucose levels (% FBGL) from Day 0 to Day 8 were plotted in FIG. 12, which allows the bioactivity of a fusion protein to be determined. FIG. 12 demonstrates that the homodimer of the SEQ ID NO: 32 can lower blood glucose for a significant period of time on a single dose.

Example 29: In Vivo Pharmacodynamics (PD) after a Single Administration of an Insulin Fc-Fusion Protein in Felines

[0178] A bioactive insulin-Fc fusion homodimer construct of SEQ ID NO: 32 was synthesized according to Example 1 and was assessed for its effects on fasting blood glucose levels as follows. N=3 healthy, antibody-naïve, cats weighing approximately 5 kg were used. On Day 0, the cats received a single injection of a pharmaceutical composition containing an insulin Fc-fusion protein homodimer of SEQ ID NO: 32 in a solution of 50 mM sodium hydrogen phosphate, 150 mM sodium chloride, and 0.02% v/v Tween-80 at pH 7.5, at a dose of 6 nmol/kg (equivalent to 0.39 mg Fc fusion protein/kg or 1.9 U/kg insulin equivalent on molar basis). On Day 0 immediately prior to injection and at 15, 30, 45, 60, 120, 240, 360, and 480 minutes and at 1, 2, 3, 4, 5, 6, 7, and 8 days post injection blood was collected from a suitable vein.

[0179] For each time point, a minimum of 1 mL of whole blood was collected. A glucose level reading was immediately determined using a glucose meter (ACCU-CHEK®

Aviva Plus), which requires approximately one drop of blood. Average % fasting blood glucose levels (% FBGL) from Day 0 to Day 8 were plotted in FIG. 12, which allows the bioactivity of a fusion protein to be determined. FIG. 12 demonstrates that the homodimer of the SEQ ID NO: 32 can lower blood glucose for a significant period of time on a single dose.

Example 29: In Vivo Pharmacodynamics (PD) after a Single Administration of an Insulin Fc-Fusion Protein in Felines

[0180] A bioactive insulin-Fc fusion homodimer construct is synthesized according to Example 1 and is assessed for its effects on fasting blood glucose levels as follows. Healthy, antibody-naïve, cats weighing approximately 5 kg are used. On Day 0, the cats receive a single injection of a pharmaceutical composition containing an insulin Fc-fusion protein homodimer in a solution of 50 mM sodium hydrogen phosphate, 150 mM sodium chloride, and 0.02% v/v Tween-80 at pH 7.5, at a dose of 6 nmol/kg (equivalent to 0.39 mg Fc fusion protein/kg or 1.9 U/kg insulin equivalent on molar basis). On Day 0 immediately prior to injection and at 15, 30, 45, 60, 120, 240, 360, and 480 minutes and at 1, 2, 3, 4, 5, 6, 7, and 8 days post injection blood was collected from a suitable vein.

[0181] For each time point, a minimum of 1 mL of whole blood is collected. A glucose level reading is immediately determined using a glucose meter (ACCU-CHEK® Aviva Plus), which requires approximately one drop of blood. Average % fasting blood glucose levels (% FBGL) from Day 0 to Day 8 are plotted, which allows the bioactivity of a fusion protein to be determined. It is expected that the homodimers of SEQ ID NO: 34 and SEQ ID NO: 36 will lower blood glucose for a significant period of time on a single dose.

Example 30: In Vivo Immunogenicity Screening after Repeated Subcutaneous Doses of the Feline Insulin-Fc Fusion Proteins of SEQ ID NO: 32, SEQ ID NO: 34, and SEQ ID NO: 36

[0182] The feline insulin-Fc fusion protein configurations of SEQ ID NO: 32, SEQ ID NO: 34 and SEQ ID NO: 36 are evaluated for in vivo immunogenicity after repeated subcutaneous doses.

[0183] The insulin-Fc fusion protein configurations are manufactured in HEK293 cells according to Example 2 and purified using a Protein A column according to Example 4. The structures are confirmed according to Example 6 by non-reducing and reducing CE-SDS, and the sequences are further identified by LC-MS with glycan removal according to Example 8. The % homodimers are measured by size-exclusion chromatography according to Example 10. The in vivo bioactivity after a single dose of the insulin-Fc fusion protein configuration being tested is measured according to Example 14 and the in vivo bioactivity is measured after repeated doses according to Example 16.

[0184] It is expected that the feline insulin-Fc fusion protein configuration of SEQ ID NO: 32 (comprising the Fc fragment of SEQ ID NO: 19) and the feline insulin-Fc fusion protein configuration of SEQ ID NO: 34 (comprising the Fc fragment of SEQ ID NO: 20) will exhibit decreasing bioactivity in vivo after repeated doses. That is, the NAOC and the NAOCR do not maintain an NAOCR greater than 0.6

over at least three doses. It is further expected that the feline insulin-Fc fusion protein configuration of SEQ ID NO: 36 (comprising the Fc fragment of SEQ ID NO: 21 with the asparagine to lysine mutation at the cNg location) will exhibit sustained bioactivity in vivo after repeated doses. That is, the NAOC and the NAOCR do maintain an NAOCR greater than 0.6 over at least three doses.

Example 31: Exemplary Insulin-Fc Fusion Protein Domains and Sequences

[0185] Exemplary insulin-Fc fusion protein domains and sequences used in the above Examples are shown in FIG. 4.

EQUIVALENTS

[0186] In the claims articles such as “a,” “an,” and “the” may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include “or” between one or more members of a group are considered satisfied if one, more than one, or all the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The disclosure includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The disclosure includes embodiments in which more than one, or all the group members are present in, employed in, or otherwise relevant to a given product or process.

[0187] Furthermore, the disclosure encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, and descriptive terms from one or more of the listed claims are introduced into another claim. For example, any claim that is dependent on another claim can be modified to include one or more limitations found in any other claim that is dependent on the same base claim. Where elements are presented as lists, e.g., in Markush group format, each subgroup of the elements is also disclosed, and any element(s) can be removed from the group. In general, where the disclosure, or aspects of the disclosure, is/are referred to as comprising elements and/or features, certain embodiments of the disclosure or aspects of the disclosure consist, or consist essentially of, such elements and/or features. For purposes of simplicity, those embodiments have not been specifically set forth in haec verba herein. It is also noted that the terms “comprise(s),” “comprising,” “contain(s),” and “containing” are intended to be open and the use thereof permits the inclusion of additional elements or steps. Where ranges are given, endpoints are included. Furthermore, unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or sub-range within the stated ranges in different embodiments of the disclosure, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

[0188] Additional advantages of the various embodiments of the technology will be apparent to those skilled in the art upon review of the disclosure herein and the working examples. It will be appreciated that the various embodiments described herein are not necessarily mutually exclusive unless otherwise indicated herein. For example, a feature described or depicted in one embodiment may also be included in other embodiments, but is not necessarily

included. Thus, the present invention encompasses a variety of combinations and/or integrations of the specific embodiments described herein.

[0189] The present description also uses numerical ranges to quantify certain parameters relating to various embodiments of the invention. It should be understood that when numerical ranges are provided, such ranges are to be con-

strued as providing literal support for claim limitations that only recite the lower value of the range as well as claim limitations that only recite the upper value of the range. For example, a disclosed numerical range of about 10 to about 100 provides literal support for a claim reciting “greater than about 10” (with no upper bounds) and a claim reciting “less than about 100” (with no lower bounds).

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 39

<210> SEQ ID NO 1
 <211> LENGTH: 30
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens
 <220> FEATURE:
 <221> NAME/KEY: Human Insulin B-Chain
 <222> LOCATION: (1)..(30)

<400> SEQUENCE: 1

```
Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr
1           5           10           15
Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr
                20           25           30
```

<210> SEQ ID NO 2
 <211> LENGTH: 21
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens
 <220> FEATURE:
 <221> NAME/KEY: Human Insulin A-Chain
 <222> LOCATION: (1)..(21)

<400> SEQUENCE: 2

```
Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu
1           5           10           15
Glu Asn Tyr Cys Asn
                20
```

<210> SEQ ID NO 3
 <211> LENGTH: 86
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens
 <220> FEATURE:
 <221> NAME/KEY: Human ProInsulin
 <222> LOCATION: (1)..(86)

<400> SEQUENCE: 3

```
Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr
1           5           10           15
Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Arg Arg
                20           25           30
Glu Ala Glu Asp Leu Gln Val Gly Gln Val Glu Leu Gly Gly Gly Pro
                35           40           45
Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu Glu Gly Ser Leu Gln Lys
                50           55           60
Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln
                65           70           75           80
Leu Glu Asn Tyr Cys Asn
                85
```

-continued

```

<210> SEQ ID NO 4
<211> LENGTH: 57
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Insulin Polypeptide

<400> SEQUENCE: 4

Phe Val Asn Gln His Leu Cys Gly Ser Asp Leu Val Glu Ala Leu Tyr
1           5           10           15

Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Asp Pro Thr Gly Gly
                20           25           30

Gly Pro Arg Arg Gly Ile Val Glu Gln Cys Cys His Ser Ile Cys Ser
            35           40           45

Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
50           55

```

```

<210> SEQ ID NO 5
<211> LENGTH: 57
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Insulin Polypeptide

<400> SEQUENCE: 5

Phe Val Asn Gln His Leu Cys Gly Ser Asp Leu Val Glu Ala Leu Ala
1           5           10           15

Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Asp Pro Thr Gly Gly
                20           25           30

Gly Pro Arg Arg Gly Ile Val Glu Gln Cys Cys His Ser Ile Cys Ser
            35           40           45

Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
50           55

```

```

<210> SEQ ID NO 6
<211> LENGTH: 58
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Insulin Polypeptide

<400> SEQUENCE: 6

Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr
1           5           10           15

Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ala Ala Ala
                20           25           30

Ala Ala Ala Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys
            35           40           45

Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
50           55

```

```

<210> SEQ ID NO 7
<211> LENGTH: 57
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Insulin Polypeptide

<400> SEQUENCE: 7

Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr

```


-continued

1	5	10	15
Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ala Gly Gly	20	25	30
Gly Pro Arg Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser	35	40	45
Leu Tyr Gln Leu Glu Asn Tyr Cys Asn	50	55	

<210> SEQ ID NO 8
 <211> LENGTH: 6
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Synthetic C-chain

<400> SEQUENCE: 8

Gly Gly Gly Pro Arg Arg
 1 5

<210> SEQ ID NO 9
 <211> LENGTH: 9
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Synthetic Linker

<400> SEQUENCE: 9

Gly Gly Gly Gly Ala Gly Gly Gly Gly
 1 5

<210> SEQ ID NO 10
 <211> LENGTH: 9
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Synthetic Linker

<400> SEQUENCE: 10

Gly Gly Gly Gly Ser Gly Gly Gly Gly
 1 5

<210> SEQ ID NO 11
 <211> LENGTH: 228
 <212> TYPE: PRT
 <213> ORGANISM: Canis familiaris

<400> SEQUENCE: 11

Arg Cys Thr Asp Thr Pro Pro Cys Pro Val Pro Glu Pro Leu Gly Gly	5	10	15
Pro Ser Val Leu Ile Phe Pro Pro Lys Pro Lys Asp Ile Leu Arg Ile	20	25	30
Thr Arg Thr Pro Glu Val Thr Cys Val Val Leu Asp Leu Gly Arg Glu	35	40	45
Asp Pro Glu Val Gln Ile Ser Trp Phe Val Asp Gly Lys Glu Val His	50	55	60
Thr Ala Lys Thr Gln Ser Arg Glu Gln Gln Phe Asn Gly Thr Tyr Arg	65	70	75
Val Val Ser Val Leu Pro Ile Glu His Gln Asp Trp Leu Thr Gly Lys	85	90	95

-continued

Glu Phe Lys Cys Arg Val Asn His Ile Asp Leu Pro Ser Pro Ile Glu
 100 105 110
 Arg Thr Ile Ser Lys Ala Arg Gly Arg Ala His Lys Pro Ser Val Tyr
 115 120 125
 Val Leu Pro Pro Ser Pro Lys Glu Leu Ser Ser Ser Asp Thr Val Ser
 130 135 140
 Ile Thr Cys Leu Ile Lys Asp Phe Tyr Pro Pro Asp Ile Asp Val Glu
 145 150 155 160
 Trp Gln Ser Asn Gly Gln Gln Glu Pro Glu Arg Lys His Arg Met Thr
 165 170 175
 Pro Pro Gln Leu Asp Glu Asp Gly Ser Tyr Phe Leu Tyr Ser Lys Leu
 180 185 190
 Ser Val Asp Lys Ser Arg Trp Gln Gln Gly Asp Pro Phe Thr Cys Ala
 195 200 205
 Val Met His Glu Thr Leu Gln Asn His Tyr Thr Asp Leu Ser Leu Ser
 210 215 220
 His Ser Pro Gly
 225

<210> SEQ ID NO 12
 <211> LENGTH: 224
 <212> TYPE: PRT
 <213> ORGANISM: Canis familiaris

<400> SEQUENCE: 12

Asp Cys Pro Lys Cys Pro Ala Pro Glu Met Leu Gly Gly Pro Ser Val
 1 5 10 15
 Phe Ile Phe Pro Pro Lys Pro Lys Asp Thr Leu Leu Ile Ala Arg Thr
 20 25 30
 Pro Glu Val Thr Cys Val Val Val Asp Leu Asp Pro Glu Asp Pro Glu
 35 40 45
 Val Gln Ile Ser Trp Phe Val Asp Gly Lys Gln Met Gln Thr Ala Lys
 50 55 60
 Thr Gln Pro Arg Glu Glu Gln Phe Asn Gly Thr Tyr Arg Val Val Ser
 65 70 75 80
 Val Leu Pro Ile Gly His Gln Asp Trp Leu Lys Gly Lys Gln Phe Thr
 85 90 95
 Cys Lys Val Asn Asn Lys Ala Leu Pro Ser Pro Ile Glu Arg Thr Ile
 100 105 110
 Ser Lys Ala Arg Gly Gln Ala His Gln Pro Ser Val Tyr Val Leu Pro
 115 120 125
 Pro Ser Arg Glu Glu Leu Ser Lys Asn Thr Val Ser Leu Thr Cys Leu
 130 135 140
 Ile Lys Asp Phe Phe Pro Pro Asp Ile Asp Val Glu Trp Gln Ser Asn
 145 150 155 160
 Gly Gln Gln Glu Pro Glu Ser Lys Tyr Arg Thr Thr Pro Pro Gln Leu
 165 170 175
 Asp Glu Asp Gly Ser Tyr Phe Leu Tyr Ser Lys Leu Ser Val Asp Lys
 180 185 190
 Ser Arg Trp Gln Arg Gly Asp Thr Phe Ile Cys Ala Val Met His Glu
 195 200 205
 Ala Leu His Asn His Tyr Thr Gln Glu Ser Leu Ser His Ser Pro Gly
 210 215 220

-continued

```

<210> SEQ ID NO 13
<211> LENGTH: 225
<212> TYPE: PRT
<213> ORGANISM: Canis familiaris

<400> SEQUENCE: 13

Cys Asn Asn Cys Pro Cys Pro Gly Cys Gly Leu Leu Gly Gly Pro Ser
1          5          10          15
Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Ile Leu Val Thr Ala Arg
20          25          30
Thr Pro Thr Val Thr Cys Val Val Val Asp Leu Asp Pro Glu Asn Pro
35          40          45
Glu Val Gln Ile Ser Trp Phe Val Asp Ser Lys Gln Val Gln Thr Ala
50          55          60
Asn Thr Gln Pro Arg Glu Glu Gln Ser Asn Gly Thr Tyr Arg Val Val
65          70          75          80
Ser Val Leu Pro Ile Gly His Gln Asp Trp Leu Ser Gly Lys Gln Phe
85          90          95
Lys Cys Lys Val Asn Asn Lys Ala Leu Pro Ser Pro Ile Glu Glu Ile
100         105         110
Ile Ser Lys Thr Pro Gly Gln Ala His Gln Pro Asn Val Tyr Val Leu
115         120         125
Pro Pro Ser Arg Asp Glu Met Ser Lys Asn Thr Val Thr Leu Thr Cys
130         135         140
Leu Val Lys Asp Phe Phe Pro Pro Glu Ile Asp Val Glu Trp Gln Ser
145         150         155         160
Asn Gly Gln Gln Glu Pro Glu Ser Lys Tyr Arg Met Thr Pro Pro Gln
165         170         175
Leu Asp Glu Asp Gly Ser Tyr Phe Leu Tyr Ser Lys Leu Ser Val Asp
180         185         190
Lys Ser Arg Trp Gln Arg Gly Asp Thr Phe Ile Cys Ala Val Met His
195         200         205
Glu Ala Leu His Asn His Tyr Thr Gln Ile Ser Leu Ser His Ser Pro
210         215         220

Gly
225

```

```

<210> SEQ ID NO 14
<211> LENGTH: 225
<212> TYPE: PRT
<213> ORGANISM: Canis familiaris

<400> SEQUENCE: 14

Cys Ile Ser Pro Cys Pro Val Pro Glu Ser Leu Gly Gly Pro Ser Val
1          5          10          15
Phe Ile Phe Pro Pro Lys Pro Lys Asp Ile Leu Arg Ile Thr Arg Thr
20          25          30
Pro Glu Ile Thr Cys Val Val Leu Asp Leu Gly Arg Glu Asp Pro Glu
35          40          45
Val Gln Ile Ser Trp Phe Val Asp Gly Lys Glu Val His Thr Ala Lys
50          55          60
Thr Gln Pro Arg Glu Gln Gln Phe Asn Ser Thr Tyr Arg Val Val Ser
65          70          75          80

```

-continued

Val Leu Pro Ile Glu His Gln Asp Trp Leu Thr Gly Lys Glu Phe Lys
85 90 95

Cys Arg Val Asn His Ile Gly Leu Pro Ser Pro Ile Glu Arg Thr Ile
100 105 110

Ser Lys Ala Arg Gly Gln Ala His Gln Pro Ser Val Tyr Val Leu Pro
115 120 125

Pro Ser Pro Lys Glu Leu Ser Ser Ser Asp Thr Val Thr Leu Thr Cys
130 135 140

Leu Ile Lys Asp Phe Phe Pro Pro Glu Ile Asp Val Glu Trp Gln Ser
145 150 155 160

Asn Gly Gln Pro Glu Pro Glu Ser Lys Tyr His Thr Thr Ala Pro Gln
165 170 175

Leu Asp Glu Asp Gly Ser Tyr Phe Leu Tyr Ser Lys Leu Ser Val Asp
180 185 190

Lys Ser Arg Trp Gln Gln Gly Asp Thr Phe Thr Cys Ala Val Met His
195 200 205

Glu Ala Leu Gln Asn His Tyr Thr Asp Leu Ser Leu Ser His Ser Pro
210 215 220

Gly
225

<210> SEQ ID NO 15
<211> LENGTH: 224
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Fc Fragment

<400> SEQUENCE: 15

Asp Cys Pro Lys Cys Pro Ala Pro Glu Met Leu Gly Gly Pro Ser Val
1 5 10 15

Phe Ile Phe Pro Pro Lys Pro Lys Asp Thr Leu Leu Ile Ala Arg Thr
20 25 30

Pro Glu Val Thr Cys Val Val Val Asp Leu Asp Pro Glu Asp Pro Glu
35 40 45

Val Gln Ile Ser Trp Phe Val Asp Gly Lys Gln Met Gln Thr Ala Lys
50 55 60

Thr Gln Pro Arg Glu Glu Gln Phe Ser Gly Thr Tyr Arg Val Val Ser
65 70 75 80

Val Leu Pro Ile Gly His Gln Asp Trp Leu Lys Gly Lys Gln Phe Thr
85 90 95

Cys Lys Val Asn Asn Lys Ala Leu Pro Ser Pro Ile Glu Arg Thr Ile
100 105 110

Ser Lys Ala Arg Gly Gln Ala His Gln Pro Ser Val Tyr Val Leu Pro
115 120 125

Pro Ser Arg Glu Glu Leu Ser Lys Asn Thr Val Ser Leu Thr Cys Leu
130 135 140

Ile Lys Asp Phe Phe Pro Pro Asp Ile Asp Val Glu Trp Gln Ser Asn
145 150 155 160

Gly Gln Gln Glu Pro Glu Ser Lys Tyr Arg Thr Thr Pro Pro Gln Leu
165 170 175

Asp Glu Asp Gly Ser Tyr Phe Leu Tyr Ser Lys Leu Ser Val Asp Lys
180 185 190

-continued

Ser Arg Trp Gln Arg Gly Asp Thr Phe Ile Cys Ala Val Met His Glu
 195 200 205
 Ala Leu His Asn His Tyr Thr Gln Glu Ser Leu Ser His Ser Pro Gly
 210 215 220

<210> SEQ ID NO 16
 <211> LENGTH: 223
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Synthetic Fc Fragment

<400> SEQUENCE: 16

Cys Pro Lys Cys Pro Ala Pro Glu Met Leu Gly Gly Pro Ser Val Phe
 1 5 10 15
 Ile Phe Pro Pro Lys Pro Lys Asp Thr Leu Leu Ile Ala Arg Thr Pro
 20 25 30
 Glu Val Thr Cys Val Val Val Asp Leu Asp Pro Glu Asp Pro Glu Val
 35 40 45
 Gln Ile Ser Trp Phe Val Asp Gly Lys Gln Met Gln Thr Ala Lys Thr
 50 55 60
 Gln Pro Arg Glu Glu Gln Phe Lys Gly Thr Tyr Arg Val Val Ser Val
 65 70 75 80
 Leu Pro Ile Gly His Gln Asp Trp Leu Lys Gly Lys Gln Phe Thr Cys
 85 90 95
 Lys Val Asn Asn Lys Ala Leu Pro Ser Pro Ile Glu Arg Thr Ile Ser
 100 105 110
 Lys Ala Arg Gly Gln Ala His Gln Pro Ser Val Tyr Val Leu Pro Pro
 115 120 125
 Ser Arg Glu Glu Leu Ser Lys Asn Thr Val Ser Leu Thr Cys Leu Ile
 130 135 140
 Lys Asp Phe Phe Pro Pro Asp Ile Asp Val Glu Trp Gln Ser Asn Gly
 145 150 155 160
 Gln Gln Glu Pro Glu Ser Lys Tyr Arg Thr Thr Pro Pro Gln Leu Asp
 165 170 175
 Glu Asp Gly Ser Tyr Phe Leu Tyr Ser Lys Leu Ser Val Asp Lys Ser
 180 185 190
 Arg Trp Gln Arg Gly Asp Thr Phe Ile Cys Ala Val Met His Glu Ala
 195 200 205
 Leu His Asn His Tyr Thr Gln Glu Ser Leu Ser His Ser Pro Gly
 210 215 220

<210> SEQ ID NO 17
 <211> LENGTH: 224
 <212> TYPE: PRT
 <213> ORGANISM: Felis catus

<400> SEQUENCE: 17

Asp Cys Pro Lys Cys Pro Pro Pro Glu Met Leu Gly Gly Pro Ser Ile
 1 5 10 15
 Phe Ile Phe Pro Pro Lys Pro Lys Asp Thr Leu Ser Ile Ser Arg Thr
 20 25 30
 Pro Glu Val Thr Cys Leu Val Val Asp Leu Gly Pro Asp Asp Ser Asp
 35 40 45

-continued

```

Val Gln Ile Thr Trp Phe Val Asp Asn Thr Gln Val Tyr Thr Ala Lys
 50                               55                               60

Thr Ser Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr Arg Val Val Ser
65                               70                               75                               80

Val Leu Pro Ile Leu His Gln Asp Trp Leu Lys Gly Lys Glu Phe Lys
                               85                               90                               95

Cys Lys Val Asn Ser Lys Ser Leu Pro Ser Pro Ile Glu Arg Thr Ile
                               100                              105                              110

Ser Lys Ala Lys Gly Gln Pro His Glu Pro Gln Val Tyr Val Leu Pro
                               115                              120                              125

Pro Ala Gln Glu Glu Leu Ser Arg Asn Lys Val Ser Val Thr Cys Leu
                               130                              135                              140

Ile Lys Ser Phe His Pro Pro Asp Ile Ala Val Glu Trp Glu Ile Thr
145                               150                              155                              160

Gly Gln Pro Glu Pro Glu Asn Asn Tyr Arg Thr Thr Pro Pro Gln Leu
                               165                              170                              175

Asp Ser Asp Gly Thr Tyr Phe Val Tyr Ser Lys Leu Ser Val Asp Arg
                               180                              185                              190

Ser His Trp Gln Arg Gly Asn Thr Tyr Thr Cys Ser Val Ser His Glu
                               195                              200                              205

Ala Leu His Ser His His Thr Gln Lys Ser Leu Thr Gln Ser Pro Gly
                               210                              215                              220
    
```

```

<210> SEQ ID NO 18
<211> LENGTH: 224
<212> TYPE: PRT
<213> ORGANISM: Felis catus
    
```

<400> SEQUENCE: 18

```

Asp Cys Pro Lys Cys Pro Pro Pro Glu Met Leu Gly Gly Pro Ser Ile
1                               5                               10                               15

Phe Ile Phe Pro Pro Lys Pro Lys Asp Thr Leu Ser Ile Ser Arg Thr
                               20                               25                               30

Pro Glu Val Thr Cys Leu Val Val Asp Leu Gly Pro Asp Asp Ser Asp
                               35                               40                               45

Val Gln Ile Thr Trp Phe Val Asp Asn Thr Gln Val Tyr Thr Ala Lys
50                               55                               60

Thr Ser Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr Arg Val Val Ser
65                               70                               75                               80

Val Leu Pro Ile Leu His Gln Asp Trp Leu Lys Gly Lys Glu Phe Lys
                               85                               90                               95

Cys Lys Val Asn Ser Lys Ser Leu Pro Ser Pro Ile Glu Arg Thr Ile
                               100                              105                              110

Ser Lys Asp Lys Gly Gln Pro His Glu Pro Gln Val Tyr Val Leu Pro
                               115                              120                              125

Pro Ala Gln Glu Glu Leu Ser Arg Asn Lys Val Ser Val Thr Cys Leu
                               130                              135                              140

Ile Glu Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ile Thr
145                               150                              155                              160

Gly Gln Pro Glu Pro Glu Asn Asn Tyr Arg Thr Thr Pro Pro Gln Leu
                               165                              170                              175

Asp Ser Asp Gly Thr Tyr Phe Leu Tyr Ser Arg Leu Ser Val Asp Arg
                               180                              185                              190
    
```

-continued

Ser Arg Trp Gln Arg Gly Asn Thr Tyr Thr Cys Ser Val Ser His Glu
195 200 205

Ala Leu His Ser His His Thr Gln Lys Ser Leu Thr Gln Ser Pro Gly
210 215 220

<210> SEQ ID NO 19
<211> LENGTH: 225
<212> TYPE: PRT
<213> ORGANISM: Felis catus

<400> SEQUENCE: 19

Gly Glu Gly Pro Lys Cys Pro Val Pro Glu Ile Pro Gly Ala Pro Ser
1 5 10 15

Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Thr Leu Ser Ile Ser Arg
20 25 30

Thr Pro Glu Val Thr Cys Leu Val Val Asp Leu Gly Pro Asp Asp Ser
35 40 45

Asn Val Gln Ile Thr Trp Phe Val Asp Asn Thr Glu Met His Thr Ala
50 55 60

Lys Thr Arg Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr Arg Val Val
65 70 75 80

Ser Val Leu Pro Ile Leu His Gln Asp Trp Leu Lys Gly Lys Glu Phe
85 90 95

Lys Cys Lys Val Asn Ser Lys Ser Leu Pro Ser Ala Met Glu Arg Thr
100 105 110

Ile Ser Lys Ala Lys Gly Gln Pro His Glu Pro Gln Val Tyr Val Leu
115 120 125

Pro Pro Thr Gln Glu Glu Leu Ser Glu Asn Lys Val Ser Val Thr Cys
130 135 140

Leu Ile Lys Gly Phe His Pro Pro Asp Ile Ala Val Glu Trp Glu Ile
145 150 155 160

Thr Gly Gln Pro Glu Pro Glu Asn Asn Tyr Gln Thr Thr Pro Pro Gln
165 170 175

Leu Asp Ser Asp Gly Thr Tyr Phe Leu Tyr Ser Arg Leu Ser Val Asp
180 185 190

Arg Ser His Trp Gln Arg Gly Asn Thr Tyr Thr Cys Ser Val Ser His
195 200 205

Glu Ala Leu His Ser His His Thr Gln Lys Ser Leu Thr Gln Ser Pro
210 215 220

Gly
225

<210> SEQ ID NO 20
<211> LENGTH: 225
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Fc Fragment

<400> SEQUENCE: 20

Gly Glu Gly Pro Lys Cys Pro Val Pro Glu Ile Pro Gly Ala Pro Ser
1 5 10 15

Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Thr Leu Ser Ile Ser Arg
20 25 30

-continued

```

Thr Pro Glu Val Thr Cys Leu Val Val Asp Leu Gly Pro Asp Asp Ser
    35                               40                               45

Asn Val Gln Ile Thr Trp Phe Val Asp Asn Thr Glu Met His Thr Ala
    50                               55                               60

Lys Thr Arg Pro Arg Glu Glu Gln Phe Ser Ser Thr Tyr Arg Val Val
    65                               70                               75                               80

Ser Val Leu Pro Ile Leu His Gln Asp Trp Leu Lys Gly Lys Glu Phe
    85                               90                               95

Lys Cys Lys Val Asn Ser Lys Ser Leu Pro Ser Ala Met Glu Arg Thr
    100                              105                              110

Ile Ser Lys Ala Lys Gly Gln Pro His Glu Pro Gln Val Tyr Val Leu
    115                              120                              125

Pro Pro Thr Gln Glu Glu Leu Ser Glu Asn Lys Val Ser Val Thr Cys
    130                              135                              140

Leu Ile Lys Gly Phe His Pro Pro Asp Ile Ala Val Glu Trp Glu Ile
    145                              150                              155                              160

Thr Gly Gln Pro Glu Pro Glu Asn Asn Tyr Gln Thr Thr Pro Pro Gln
    165                              170

Leu Asp Ser Asp Gly Thr Tyr Phe Leu Tyr Ser Arg Leu Ser Val Asp
    180                              185                              190

Arg Ser His Trp Gln Arg Gly Asn Thr Tyr Thr Cys Ser Val Ser His
    195                              200                              205

Glu Ala Leu His Ser His His Thr Gln Lys Ser Leu Thr Gln Ser Pro
    210                              215                              220

Gly
225

```

```

<210> SEQ ID NO 21
<211> LENGTH: 225
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Fc Fragment

```

```

<400> SEQUENCE: 21

```

```

Gly Glu Gly Pro Lys Cys Pro Val Pro Glu Ile Pro Gly Ala Pro Ser
1      5      10      15

Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Thr Leu Ser Ile Ser Arg
    20      25      30

Thr Pro Glu Val Thr Cys Leu Val Val Asp Leu Gly Pro Asp Asp Ser
    35      40      45

Asn Val Gln Ile Thr Trp Phe Val Asp Asn Thr Glu Met His Thr Ala
    50      55      60

Lys Thr Arg Pro Arg Glu Glu Gln Phe Lys Ser Thr Tyr Arg Val Val
    65      70      75      80

Ser Val Leu Pro Ile Leu His Gln Asp Trp Leu Lys Gly Lys Glu Phe
    85      90      95

Lys Cys Lys Val Asn Ser Lys Ser Leu Pro Ser Ala Met Glu Arg Thr
    100     105     110

Ile Ser Lys Ala Lys Gly Gln Pro His Glu Pro Gln Val Tyr Val Leu
    115     120     125

Pro Pro Thr Gln Glu Glu Leu Ser Glu Asn Lys Val Ser Val Thr Cys
    130     135     140

```


-continued

Leu Ile Lys Gly Phe His Pro Pro Asp Ile Ala Val Glu Trp Glu Ile
 145 150 155 160

Thr Gly Gln Pro Glu Pro Glu Asn Asn Tyr Gln Thr Thr Pro Pro Gln
 165 170 175

Leu Asp Ser Asp Gly Thr Tyr Phe Leu Tyr Ser Arg Leu Ser Val Asp
 180 185 190

Arg Ser His Trp Gln Arg Gly Asn Thr Tyr Thr Cys Ser Val Ser His
 195 200 205

Glu Ala Leu His Ser His His Thr Gln Lys Ser Leu Thr Gln Ser Pro
 210 215 220

Gly
 225

<210> SEQ ID NO 22
 <211> LENGTH: 294
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Synthetic Insulin-Fc Fusion Protein

<400> SEQUENCE: 22

Phe Val Asn Gln His Leu Cys Gly Ser Asp Leu Val Glu Ala Leu Ala
 1 5 10 15

Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Asp Pro Thr Gly Gly
 20 25 30

Gly Pro Arg Arg Gly Ile Val Glu Gln Cys Cys His Ser Ile Cys Ser
 35 40 45

Leu Tyr Gln Leu Glu Asn Tyr Cys Asn Gly Gly Gly Ala Gly Gly
 50 55 60

Gly Gly Arg Cys Thr Asp Thr Pro Pro Cys Pro Val Pro Glu Pro Leu
 65 70 75 80

Gly Gly Pro Ser Val Leu Ile Phe Pro Pro Lys Pro Lys Asp Ile Leu
 85 90 95

Arg Ile Thr Arg Thr Pro Glu Val Thr Cys Val Val Leu Asp Leu Gly
 100 105 110

Arg Glu Asp Pro Glu Val Gln Ile Ser Trp Phe Val Asp Gly Lys Glu
 115 120 125

Val His Thr Ala Lys Thr Gln Ser Arg Glu Gln Gln Phe Asn Gly Thr
 130 135 140

Tyr Arg Val Val Ser Val Leu Pro Ile Glu His Gln Asp Trp Leu Thr
 145 150 155 160

Gly Lys Glu Phe Lys Cys Arg Val Asn His Ile Asp Leu Pro Ser Pro
 165 170 175

Ile Glu Arg Thr Ile Ser Lys Ala Arg Gly Arg Ala His Lys Pro Ser
 180 185 190

Val Tyr Val Leu Pro Pro Ser Pro Lys Glu Leu Ser Ser Ser Asp Thr
 195 200 205

Val Ser Ile Thr Cys Leu Ile Lys Asp Phe Tyr Pro Pro Asp Ile Asp
 210 215 220

Val Glu Trp Gln Ser Asn Gly Gln Gln Glu Pro Glu Arg Lys His Arg
 225 230 235 240

Met Thr Pro Pro Gln Leu Asp Glu Asp Gly Ser Tyr Phe Leu Tyr Ser
 245 250 255

-continued

Lys Leu Ser Val Asp Lys Ser Arg Trp Gln Gln Gly Asp Pro Phe Thr
 260 265 270

Cys Ala Val Met His Glu Thr Leu Gln Asn His Tyr Thr Asp Leu Ser
 275 280 285

Leu Ser His Ser Pro Gly
 290

<210> SEQ ID NO 23
 <211> LENGTH: 294
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Synthetic Insulin-Fc Fusion Protein

<400> SEQUENCE: 23

Phe Val Asn Gln His Leu Cys Gly Ser Asp Leu Val Glu Ala Leu Tyr
 1 5 10 15

Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Asp Pro Thr Gly Gly
 20 25 30

Gly Pro Arg Arg Gly Ile Val Glu Gln Cys Cys His Ser Ile Cys Ser
 35 40 45

Leu Tyr Gln Leu Glu Asn Tyr Cys Asn Gly Gly Gly Ser Gly Gly
 50 55 60

Gly Gly Arg Cys Thr Asp Thr Pro Pro Cys Pro Val Pro Glu Pro Leu
 65 70 75 80

Gly Gly Pro Ser Val Leu Ile Phe Pro Pro Lys Pro Lys Asp Ile Leu
 85 90 95

Arg Ile Thr Arg Thr Pro Glu Val Thr Cys Val Val Leu Asp Leu Gly
 100 105 110

Arg Glu Asp Pro Glu Val Gln Ile Ser Trp Phe Val Asp Gly Lys Glu
 115 120 125

Val His Thr Ala Lys Thr Gln Ser Arg Glu Gln Gln Phe Asn Gly Thr
 130 135 140

Tyr Arg Val Val Ser Val Leu Pro Ile Glu His Gln Asp Trp Leu Thr
 145 150 155 160

Gly Lys Glu Phe Lys Cys Arg Val Asn His Ile Asp Leu Pro Ser Pro
 165 170 175

Ile Glu Arg Thr Ile Ser Lys Ala Arg Gly Arg Ala His Lys Pro Ser
 180 185 190

Val Tyr Val Leu Pro Pro Ser Pro Lys Glu Leu Ser Ser Ser Asp Thr
 195 200 205

Val Ser Ile Thr Cys Leu Ile Lys Asp Phe Tyr Pro Pro Asp Ile Asp
 210 215 220

Val Glu Trp Gln Ser Asn Gly Gln Gln Glu Pro Glu Arg Lys His Arg
 225 230 235 240

Met Thr Pro Pro Gln Leu Asp Glu Asp Gly Ser Tyr Phe Leu Tyr Ser
 245 250 255

Lys Leu Ser Val Asp Lys Ser Arg Trp Gln Gln Gly Asp Pro Phe Thr
 260 265 270

Cys Ala Val Met His Glu Thr Leu Gln Asn His Tyr Thr Asp Leu Ser
 275 280 285

Leu Ser His Ser Pro Gly
 290

-continued

```

<210> SEQ ID NO 24
<211> LENGTH: 294
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Insulin-Fc Fusion Protein

<400> SEQUENCE: 24

Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr
1          5          10          15
Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ala Gly Gly
20          25          30
Gly Pro Arg Arg Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser
35          40          45
Leu Tyr Gln Leu Glu Asn Tyr Cys Asn Gly Gly Gly Ser Gly Gly
50          55          60
Gly Gly Arg Cys Thr Asp Thr Pro Pro Cys Pro Val Pro Glu Pro Leu
65          70          75          80
Gly Gly Pro Ser Val Leu Ile Phe Pro Pro Lys Pro Lys Asp Ile Leu
85          90          95
Arg Ile Thr Arg Thr Pro Glu Val Thr Cys Val Val Leu Asp Leu Gly
100         105         110
Arg Glu Asp Pro Glu Val Gln Ile Ser Trp Phe Val Asp Gly Lys Glu
115         120         125
Val His Thr Ala Lys Thr Gln Ser Arg Glu Gln Gln Phe Asn Gly Thr
130         135         140
Tyr Arg Val Val Ser Val Leu Pro Ile Glu His Gln Asp Trp Leu Thr
145         150         155         160
Gly Lys Glu Phe Lys Cys Arg Val Asn His Ile Asp Leu Pro Ser Pro
165         170         175
Ile Glu Arg Thr Ile Ser Lys Ala Arg Gly Arg Ala His Lys Pro Ser
180         185         190
Val Tyr Val Leu Pro Pro Ser Pro Lys Glu Leu Ser Ser Ser Asp Thr
195         200         205
Val Ser Ile Thr Cys Leu Ile Lys Asp Phe Tyr Pro Pro Asp Ile Asp
210         215         220
Val Glu Trp Gln Ser Asn Gly Gln Gln Glu Pro Glu Arg Lys His Arg
225         230         235         240
Met Thr Pro Pro Gln Leu Asp Glu Asp Gly Ser Tyr Phe Leu Tyr Ser
245         250         255
Lys Leu Ser Val Asp Lys Ser Arg Trp Gln Gln Gly Asp Pro Phe Thr
260         265         270
Cys Ala Val Met His Glu Thr Leu Gln Asn His Tyr Thr Asp Leu Ser
275         280         285

Leu Ser His Ser Pro Gly
290

```

```

<210> SEQ ID NO 25
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Insulin-Fc Fusion Protein

<400> SEQUENCE: 25

```

-continued

Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr
 1 5 10 15
 Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Ala Ala Ala
 20 25 30
 Ala Ala Ala Ala Lys Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys
 35 40 45
 Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn Gly Gly Gly Ser Gly
 50 55 60
 Gly Gly Gly Arg Cys Thr Asp Thr Pro Pro Cys Pro Val Pro Glu Pro
 65 70 75 80
 Leu Gly Gly Pro Ser Val Leu Ile Phe Pro Pro Lys Pro Lys Asp Ile
 85 90 95
 Leu Arg Ile Thr Arg Thr Pro Glu Val Thr Cys Val Val Leu Asp Leu
 100 105 110
 Gly Arg Glu Asp Pro Glu Val Gln Ile Ser Trp Phe Val Asp Gly Lys
 115 120 125
 Glu Val His Thr Ala Lys Thr Gln Ser Arg Glu Gln Gln Phe Asn Gly
 130 135 140
 Thr Tyr Arg Val Val Ser Val Leu Pro Ile Glu His Gln Asp Trp Leu
 145 150 155 160
 Thr Gly Lys Glu Phe Lys Cys Arg Val Asn His Ile Asp Leu Pro Ser
 165 170 175
 Pro Ile Glu Arg Thr Ile Ser Lys Ala Arg Gly Arg Ala His Lys Pro
 180 185 190
 Ser Val Tyr Val Leu Pro Pro Ser Pro Lys Glu Leu Ser Ser Ser Asp
 195 200 205
 Thr Val Ser Ile Thr Cys Leu Ile Lys Asp Phe Tyr Pro Pro Asp Ile
 210 215 220
 Asp Val Glu Trp Gln Ser Asn Gly Gln Gln Glu Pro Glu Arg Lys His
 225 230 235 240
 Arg Met Thr Pro Pro Gln Leu Asp Glu Asp Gly Ser Tyr Phe Leu Tyr
 245 250 255
 Ser Lys Leu Ser Val Asp Lys Ser Arg Trp Gln Gln Gly Asp Pro Phe
 260 265 270
 Thr Cys Ala Val Met His Glu Thr Leu Gln Asn His Tyr Thr Asp Leu
 275 280 285
 Ser Leu Ser His Ser Pro Gly
 290 295

<210> SEQ ID NO 26

<211> LENGTH: 290

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Synthetic Insulin-Fc Fusion Protein

<400> SEQUENCE: 26

Phe Val Asn Gln His Leu Cys Gly Ser Asp Leu Val Glu Ala Leu Ala
 1 5 10 15
 Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Asp Pro Thr Gly Gly
 20 25 30
 Gly Pro Arg Arg Gly Ile Val Glu Gln Cys Cys His Ser Ile Cys Ser
 35 40 45

-continued

Leu Tyr Gln Leu Glu Asn Tyr Cys Asn Gly Gly Gly Ala Gly Gly
 50 55 60
 Gly Gly Asp Cys Pro Lys Cys Pro Ala Pro Glu Met Leu Gly Gly Pro
 65 70 75 80
 Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Thr Leu Leu Ile Ala
 85 90 95
 Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Leu Asp Pro Glu Asp
 100 105 110
 Pro Glu Val Gln Ile Ser Trp Phe Val Asp Gly Lys Gln Met Gln Thr
 115 120 125
 Ala Lys Thr Gln Pro Arg Glu Glu Gln Phe Asn Gly Thr Tyr Arg Val
 130 135 140
 Val Ser Val Leu Pro Ile Gly His Gln Asp Trp Leu Lys Gly Lys Gln
 145 150 155 160
 Phe Thr Cys Lys Val Asn Asn Lys Ala Leu Pro Ser Pro Ile Glu Arg
 165 170 175
 Thr Ile Ser Lys Ala Arg Gly Gln Ala His Gln Pro Ser Val Tyr Val
 180 185 190
 Leu Pro Pro Ser Arg Glu Glu Leu Ser Lys Asn Thr Val Ser Leu Thr
 195 200 205
 Cys Leu Ile Lys Asp Phe Phe Pro Pro Asp Ile Asp Val Glu Trp Gln
 210 215 220
 Ser Asn Gly Gln Gln Glu Pro Glu Ser Lys Tyr Arg Thr Thr Pro Pro
 225 230 235 240
 Gln Leu Asp Glu Asp Gly Ser Tyr Phe Leu Tyr Ser Lys Leu Ser Val
 245 250 255
 Asp Lys Ser Arg Trp Gln Arg Gly Asp Thr Phe Ile Cys Ala Val Met
 260 265 270
 His Glu Ala Leu His Asn His Tyr Thr Gln Glu Ser Leu Ser His Ser
 275 280 285
 Pro Gly
 290

<210> SEQ ID NO 27

<211> LENGTH: 291

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Synthetic Insulin-Fc Fusion Protein

<400> SEQUENCE: 27

Phe Val Asn Gln His Leu Cys Gly Ser Asp Leu Val Glu Ala Leu Ala
 1 5 10 15
 Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Asp Pro Thr Gly Gly
 20 25 30
 Gly Pro Arg Arg Gly Ile Val Glu Gln Cys Cys His Ser Ile Cys Ser
 35 40 45
 Leu Tyr Gln Leu Glu Asn Tyr Cys Asn Gly Gly Gly Ala Gly Gly
 50 55 60
 Gly Gly Cys Asn Asn Cys Pro Cys Pro Gly Cys Gly Leu Leu Gly Gly
 65 70 75 80
 Pro Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Ile Leu Val Thr
 85 90 95

-continued

Ala Arg Thr Pro Thr Val Thr Cys Val Val Val Asp Leu Asp Pro Glu
 100 105 110

Asn Pro Glu Val Gln Ile Ser Trp Phe Val Asp Ser Lys Gln Val Gln
 115 120 125

Thr Ala Asn Thr Gln Pro Arg Glu Glu Gln Ser Asn Gly Thr Tyr Arg
 130 135 140

Val Val Ser Val Leu Pro Ile Gly His Gln Asp Trp Leu Ser Gly Lys
 145 150 155 160

Gln Phe Lys Cys Lys Val Asn Asn Lys Ala Leu Pro Ser Pro Ile Glu
 165 170 175

Glu Ile Ile Ser Lys Thr Pro Gly Gln Ala His Gln Pro Asn Val Tyr
 180 185 190

Val Leu Pro Pro Ser Arg Asp Glu Met Ser Lys Asn Thr Val Thr Leu
 195 200 205

Thr Cys Leu Val Lys Asp Phe Phe Pro Pro Glu Ile Asp Val Glu Trp
 210 215 220

Gln Ser Asn Gly Gln Gln Glu Pro Glu Ser Lys Tyr Arg Met Thr Pro
 225 230 235 240

Pro Gln Leu Asp Glu Asp Gly Ser Tyr Phe Leu Tyr Ser Lys Leu Ser
 245 250 255

Val Asp Lys Ser Arg Trp Gln Arg Gly Asp Thr Phe Ile Cys Ala Val
 260 265 270

Met His Glu Ala Leu His Asn His Tyr Thr Gln Ile Ser Leu Ser His
 275 280 285

Ser Pro Gly
 290

<210> SEQ ID NO 28
 <211> LENGTH: 291
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Synthetic Insulin-Fc Fusion Protein

<400> SEQUENCE: 28

Phe Val Asn Gln His Leu Cys Gly Ser Asp Leu Val Glu Ala Leu Ala
 1 5 10 15

Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Asp Pro Thr Gly Gly
 20 25 30

Gly Pro Arg Arg Gly Ile Val Glu Gln Cys Cys His Ser Ile Cys Ser
 35 40 45

Leu Tyr Gln Leu Glu Asn Tyr Cys Asn Gly Gly Gly Ala Gly Gly
 50 55 60

Gly Gly Cys Ile Ser Pro Cys Pro Val Pro Glu Ser Leu Gly Gly Pro
 65 70 75 80

Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Ile Leu Arg Ile Thr
 85 90 95

Arg Thr Pro Glu Ile Thr Cys Val Val Leu Asp Leu Gly Arg Glu Asp
 100 105 110

Pro Glu Val Gln Ile Ser Trp Phe Val Asp Gly Lys Glu Val His Thr
 115 120 125

Ala Lys Thr Gln Pro Arg Glu Gln Gln Phe Asn Ser Thr Tyr Arg Val
 130 135 140

-continued

Val Ser Val Leu Pro Ile Glu His Gln Asp Trp Leu Thr Gly Lys Glu
 145 150 155 160
 Phe Lys Cys Arg Val Asn His Ile Gly Leu Pro Ser Pro Ile Glu Arg
 165 170 175
 Thr Ile Ser Lys Ala Arg Gly Gln Ala His Gln Pro Ser Val Tyr Val
 180 185 190
 Leu Pro Pro Ser Pro Lys Glu Leu Ser Ser Ser Asp Thr Val Thr Leu
 195 200 205
 Thr Cys Leu Ile Lys Asp Phe Phe Pro Pro Glu Ile Asp Val Glu Trp
 210 215 220
 Gln Ser Asn Gly Gln Pro Glu Pro Glu Ser Lys Tyr His Thr Thr Ala
 225 230 235 240
 Pro Gln Leu Asp Glu Asp Gly Ser Tyr Phe Leu Tyr Ser Lys Leu Ser
 245 250 255
 Val Asp Lys Ser Arg Trp Gln Gln Gly Asp Thr Phe Thr Cys Ala Val
 260 265 270
 Met His Glu Ala Leu Gln Asn His Tyr Thr Asp Leu Ser Leu Ser His
 275 280 285
 Ser Pro Gly
 290

<210> SEQ ID NO 29
 <211> LENGTH: 290
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Synthetic Insulin-Fc Fusion Protein

<400> SEQUENCE: 29

Phe Val Asn Gln His Leu Cys Gly Ser Asp Leu Val Glu Ala Leu Ala
 1 5 10 15
 Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Asp Pro Thr Gly Gly
 20 25 30
 Gly Pro Arg Arg Gly Ile Val Glu Gln Cys Cys His Ser Ile Cys Ser
 35 40 45
 Leu Tyr Gln Leu Glu Asn Tyr Cys Asn Gly Gly Gly Ala Gly Gly
 50 55 60
 Gly Gly Asp Cys Pro Lys Cys Pro Ala Pro Glu Met Leu Gly Gly Pro
 65 70 75 80
 Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Thr Leu Leu Ile Ala
 85 90 95
 Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Leu Asp Pro Glu Asp
 100 105 110
 Pro Glu Val Gln Ile Ser Trp Phe Val Asp Gly Lys Gln Met Gln Thr
 115 120 125
 Ala Lys Thr Gln Pro Arg Glu Glu Gln Phe Ser Gly Thr Tyr Arg Val
 130 135 140
 Val Ser Val Leu Pro Ile Gly His Gln Asp Trp Leu Lys Gly Lys Gln
 145 150 155 160
 Phe Thr Cys Lys Val Asn Asn Lys Ala Leu Pro Ser Pro Ile Glu Arg
 165 170 175
 Thr Ile Ser Lys Ala Arg Gly Gln Ala His Gln Pro Ser Val Tyr Val
 180 185 190

-continued

Gly

<210> SEQ ID NO 32
 <211> LENGTH: 291
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Synthetic Insulin-Fc Fusion Protein

 <400> SEQUENCE: 32

 Phe Val Asn Gln His Leu Cys Gly Ser Asp Leu Val Glu Ala Leu Tyr
 1 5 10 15

 Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Asp Pro Thr Gly Gly
 20 25 30

 Gly Pro Arg Arg Gly Ile Val Glu Gln Cys Cys His Ser Ile Cys Ser
 35 40 45

 Leu Tyr Gln Leu Glu Asn Tyr Cys Asn Gly Gly Gly Ser Gly Gly
 50 55 60

 Gly Gly Gly Glu Gly Pro Lys Cys Pro Val Pro Glu Ile Pro Gly Ala
 65 70 75 80

 Pro Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Thr Leu Ser Ile
 85 90 95

 Ser Arg Thr Pro Glu Val Thr Cys Leu Val Val Asp Leu Gly Pro Asp
 100 105 110

 Asp Ser Asn Val Gln Ile Thr Trp Phe Val Asp Asn Thr Glu Met His
 115 120 125

 Thr Ala Lys Thr Arg Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr Arg
 130 135 140

 Val Val Ser Val Leu Pro Ile Leu His Gln Asp Trp Leu Lys Gly Lys
 145 150 155 160

 Glu Phe Lys Cys Lys Val Asn Ser Lys Ser Leu Pro Ser Ala Met Glu
 165 170 175

 Arg Thr Ile Ser Lys Ala Lys Gly Gln Pro His Glu Pro Gln Val Tyr
 180 185 190

 Val Leu Pro Pro Thr Gln Glu Glu Leu Ser Glu Asn Lys Val Ser Val
 195 200 205

 Thr Cys Leu Ile Lys Gly Phe His Pro Pro Asp Ile Ala Val Glu Trp
 210 215 220

 Glu Ile Thr Gly Gln Pro Glu Pro Glu Asn Asn Tyr Gln Thr Thr Pro
 225 230 235 240

 Pro Gln Leu Asp Ser Asp Gly Thr Tyr Phe Leu Tyr Ser Arg Leu Ser
 245 250 255

 Val Asp Arg Ser His Trp Gln Arg Gly Asn Thr Tyr Thr Cys Ser Val
 260 265 270

 Ser His Glu Ala Leu His Ser His His Thr Gln Lys Ser Leu Thr Gln
 275 280 285

 Ser Pro Gly
 290

<210> SEQ ID NO 33
 <211> LENGTH: 933
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:

-continued

<223> OTHER INFORMATION: Synthetic cDNA for Insulin-Fc Fusion Protein

<400> SEQUENCE: 33

```

atggaatgga gctgggtctt tctcttcttc ctgtcagtaa cgactggtgt ccactccttc    60
gtgaaccagc acctgtgctg ctccgaactg gtggaagctc tgtatctcgt gtgctggcgag    120
cggggcttct tctacaccga tcccactgga ggcgggtccac gcagaggcat cgtggaacag    180
tgctgccact ccactctgctc cctgtaccag ctggaaaact actgcaatgg cggaggtggt    240
tcaggaggcg gtggaggtga gggccccaag tgccccgtgc ccgagattcc cggtgccccc    300
agcgtgttca tctttccccc aaaaccaag gacaccctga gcatcagcag gacccccgag    360
gtgacctgcc tgggtgtgga cctgggaccc gacgacagca acgtgcagat cacctggttc    420
gtggacaaca ccgagatgca caccgccaag accaggcctc gggaggagca gttcaacagc    480
acctacaggg tggtagcgt gctgcccatc ctgcaccagg actggctgaa gggcaaggag    540
ttcaagtgca agtgtaacag caagagcctg cccagcgcca tggagaggac catcagaag    600
gccaagggtc agccccacga gccccaagtg tacgtgcttc ccccgaccca ggaggagtgtg    660
agcgagaaca aagtgagcgt gacctgcctg atcaagggtt tccaccctcc cgacatcgcc    720
gtggagtggg agatcacogg ccagcctgag ccgaaaata actaccagac caccctcca    780
cagctggaca gcgacggcac ctacttctt tacagcaggc tgtctgtgga ccgaagccat    840
tggcaaaggg gcaacaccta cacctgcagc gtgagccacg aggtctctgca cagccaccac    900
accagaagt ctctgacca gagccccgga tag    933
    
```

<210> SEQ ID NO 34
 <211> LENGTH: 291
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Synthetic Insulin-Fc Fusion Protein

<400> SEQUENCE: 34

```

Phe Val Asn Gln His Leu Cys Gly Ser Asp Leu Val Glu Ala Leu Tyr
1           5           10          15
Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Asp Pro Thr Gly Gly
20          25          30
Gly Pro Arg Arg Gly Ile Val Glu Gln Cys Cys His Ser Ile Cys Ser
35          40          45
Leu Tyr Gln Leu Glu Asn Tyr Cys Asn Gly Gly Gly Gly Ser Gly Gly
50          55          60
Gly Gly Gly Glu Gly Pro Lys Cys Pro Val Pro Glu Ile Pro Gly Ala
65          70          75          80
Pro Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Thr Leu Ser Ile
85          90          95
Ser Arg Thr Pro Glu Val Thr Cys Leu Val Val Asp Leu Gly Pro Asp
100         105         110
Asp Ser Asn Val Gln Ile Thr Trp Phe Val Asp Asn Thr Glu Met His
115         120         125
Thr Ala Lys Thr Arg Pro Arg Glu Glu Gln Phe Ser Ser Thr Tyr Arg
130         135         140
Val Val Ser Val Leu Pro Ile Leu His Gln Asp Trp Leu Lys Gly Lys
145         150         155         160
    
```

-continued

Glu Phe Lys Cys Lys Val Asn Ser Lys Ser Leu Pro Ser Ala Met Glu
 165 170 175

Arg Thr Ile Ser Lys Ala Lys Gly Gln Pro His Glu Pro Gln Val Tyr
 180 185 190

Val Leu Pro Pro Thr Gln Glu Glu Leu Ser Glu Asn Lys Val Ser Val
 195 200 205

Thr Cys Leu Ile Lys Gly Phe His Pro Pro Asp Ile Ala Val Glu Trp
 210 215 220

Glu Ile Thr Gly Gln Pro Glu Pro Glu Asn Asn Tyr Gln Thr Thr Pro
 225 230 235 240

Pro Gln Leu Asp Ser Asp Gly Thr Tyr Phe Leu Tyr Ser Arg Leu Ser
 245 250 255

Val Asp Arg Ser His Trp Gln Arg Gly Asn Thr Tyr Thr Cys Ser Val
 260 265 270

Ser His Glu Ala Leu His Ser His His Thr Gln Lys Ser Leu Thr Gln
 275 280 285

Ser Pro Gly
 290

<210> SEQ ID NO 35
 <211> LENGTH: 933
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Synthetic cDNA for Insulin-Fc Fusion Protein

<400> SEQUENCE: 35

atggaatgga gctgggtcctt tctctctctc ctgtcagtaa cgactggtgt ccactccttc 60

gtgaaccagc acctgtgctg ctccgacctg gtggaagctc tgtatctcgt gtgcggcgag 120

cggggcttct tctacaccga tcccactgga ggcggctccac gcagaggcat cgtggaacag 180

tgctgccact ccactctgctc cctgtaccag ctggaaaact actgcaatgg cggaggtggt 240

tcaggaggcg gtggaggatga gggccccaag tgccccgtgc ccgagattcc cggtgcccc 300

agcgtgttca tctttcccc aaaaccaag gacaccctga gcatcagcag gacccccgag 360

gtgacctgcc tgggtggtgga cctgggaccc gacgacagca acgtgcagat cacctggttc 420

gtggacaaca ccgagatgca caccgccaag accaggcctc gggaggagca gttcagcagc 480

acctacaggg tggtagcgt gctgccccatc ctgcaccagg actggctgaa gggcaaggag 540

ttcaagtgca aggtgaacag caagagcctg cccagcgcca tggagaggac catcagcaag 600

gccaagggtc agccccacga gccccaaagt tacgtgcttc ccccgaccca ggaggagt 660

agcgagaaca aagtgagcgt gacctgcctg atcaagggt tccaccctcc cgacatcgcc 720

gtggagtggg agatcaccgg ccagcctgag ccggaaaata actaccagac caccctcca 780

cagctggaca gcgacggcac ctacttctt tacagcaggc tgtctgtgga ccgaagccat 840

tggcaaagg gcaacaccta cacctgcagc gtgagccacg aggctctgca cagccaccac 900

accagaagt ctctgaccca gagccccgga tag 933

-continued

```

<210> SEQ ID NO 36
<211> LENGTH: 291
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Insulin-Fc Fusion Protein

<400> SEQUENCE: 36

Phe Val Asn Gln His Leu Cys Gly Ser Asp Leu Val Glu Ala Leu Tyr
1           5           10           15

Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Asp Pro Thr Gly Gly
20           25           30

Gly Pro Arg Arg Gly Ile Val Glu Gln Cys Cys His Ser Ile Cys Ser
35           40           45

Leu Tyr Gln Leu Glu Asn Tyr Cys Asn Gly Gly Gly Gly Ser Gly Gly
50           55           60

Gly Gly Gly Glu Gly Pro Lys Cys Pro Val Pro Glu Ile Pro Gly Ala
65           70           75           80

Pro Ser Val Phe Ile Phe Pro Pro Lys Pro Lys Asp Thr Leu Ser Ile
85           90           95

Ser Arg Thr Pro Glu Val Thr Cys Leu Val Val Asp Leu Gly Pro Asp
100          105          110

Asp Ser Asn Val Gln Ile Thr Trp Phe Val Asp Asn Thr Glu Met His
115          120          125

Thr Ala Lys Thr Arg Pro Arg Glu Glu Gln Phe Lys Ser Thr Tyr Arg
130          135          140

Val Val Ser Val Leu Pro Ile Leu His Gln Asp Trp Leu Lys Gly Lys
145          150          155          160

Glu Phe Lys Cys Lys Val Asn Ser Lys Ser Leu Pro Ser Ala Met Glu
165          170          175

Arg Thr Ile Ser Lys Ala Lys Gly Gln Pro His Glu Pro Gln Val Tyr
180          185          190

Val Leu Pro Pro Thr Gln Glu Glu Leu Ser Glu Asn Lys Val Ser Val
195          200          205

Thr Cys Leu Ile Lys Gly Phe His Pro Pro Asp Ile Ala Val Glu Trp
210          215          220

Glu Ile Thr Gly Gln Pro Glu Pro Glu Asn Asn Tyr Gln Thr Thr Pro
225          230          235          240

Pro Gln Leu Asp Ser Asp Gly Thr Tyr Phe Leu Tyr Ser Arg Leu Ser
245          250          255

Val Asp Arg Ser His Trp Gln Arg Gly Asn Thr Tyr Thr Cys Ser Val
260          265          270

Ser His Glu Ala Leu His Ser His His Thr Gln Lys Ser Leu Thr Gln
275          280          285

Ser Pro Gly
290

```

-continued

<210> SEQ ID NO 37
 <211> LENGTH: 933
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Synthetic cDNA for Insulin-Fc Fusion Protein

 <400> SEQUENCE: 37

 atggaatgga gctgggtcctt tctctctctc ctgtcagtaa cgactggtgt ccaactcctc 60
 gtgaaccagc acctgtgctg ctcgacctg gtggaagctc tgtatctcgt gtgctgctgag 120
 cggggcctct tctacaccga tcccaactgga ggcggtccac gcagaggcat cgtggaacag 180
 tgctgccact ccatctgctc cctgtaccag ctggaaaact actgcaatgg cggaggtggt 240
 tcaggaggcg gtggagtga gggccccaag tgcccctgctc ccgagattcc cggtgccccc 300
 agcgtgttca tctttcccc aaaacccaag gacacctga gcatcagcag gacccccgag 360
 gtgacctgcc tgggtggtga cctgggacct gacgacagca acgtgcagat cacctggttc 420
 gtggacaaca ccgagatgca caccgccaag accaggcctc gggaggagca gttcaagagc 480
 acctacaggg tggtagcgt gctgccctc ctgcaaccagg actggtgaa gggcaaggag 540
 ttcaagtgca aggtgaacag caagagcctg cccagcgcca tggagaggac catcagcaag 600
 gccaaagggtc agccccacga gccccaagtg tacgtgcttc ccccgaccca ggaggagtgt 660
 agcgagaaca aagtgagcgt gacctgctg atcaagggtt tccaccctcc cgacatcgcc 720
 gtggagtggg agatcaccgg ccagcctgag ccggaaaata actaccagac caccctcca 780
 cagctggaca gcgacggcac ctacttctt tacagcaggc tgtctgtgga ccgaagccat 840
 tggcaagggt gcaacaccta cacctgcagc gtgagccacg aggtctctgca cagccaccac 900
 acccagaagt ctctgaccca gagccccgga tag 933

<210> SEQ ID NO 38
 <211> LENGTH: 19
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Synthetic Leader Sequence

<400> SEQUENCE: 38

Met Glu Trp Ser Trp Val Phe Leu Phe Phe Leu Ser Val Thr Thr Gly
 1 5 10 15

Val His Ser

<210> SEQ ID NO 39
 <211> LENGTH: 57
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Synthetic Leader Sequence Nucleic Acid

<400> SEQUENCE: 39

atggaatgga gctgggtcctt tctctctctc ctgtcagtaa cgactggtgt ccaactc 57

We claim:

1. A recombinant cell comprising a nucleic acid encoding a fusion protein, said fusion protein comprising an insulin polypeptide and an Fc fragment, wherein the insulin polypeptide and the Fc fragment are connected by a peptide linker, wherein the Fc fragment comprises the following sequence:

(SEQ ID NO: 21)
 GEGPKCPVPEIPGAPSVFIFPPKPKDLSISRTPVETCLVVDLGPDDSNVQ
 ITWFDNTEMHAKTRPREEQFKSTYRVVSVLPILHQDWLKGKKEKCKVNS
 KSLPSAMERTISKAKQPHEPQVYVLPPTQEELSENKVSVTCLIKGFHPPD
 IAVEWEITGQPEPENNYQTTPQLDSDGTYFLYSRLSVDRSHWQRGNTYTC
 SVSHEALHSHHTQKSLTQSPG,

and wherein the insulin polypeptide comprises the following sequence:

(SEQ ID NO: 4)
 FVNQHLGSDLVEALYLVCGERGFFYDPTGGGPRRGIVEQCCHSICSLYQ
 LENYCN.

2. The recombinant cell of claim 1, wherein the cell is a HEK293 cell or a CHO cell.

3. The recombinant cell of claim 1, wherein said nucleic acid comprises cDNA encoding said fusion protein, said fusion protein comprising the following sequence:

(SEQ ID NO: 36)
 FVNQHLGSDLVEALYLVCGERGFFYDPTGGGPRRGIVEQCCHSICSLYQ
 LENYCNNGGGSGGGGGEGPKCPVPEIPGAPSVFIFPPKPKDLSISRTPEV
 TCLVVDLGPDDSNVQITWFDNTEMHAKTRPREEQFKSTYRVVSVLPILH
 QDWLKGKFEKCKVNSKSLPSAMERTISKAKQPHEPQVYVLPPTQEELSEN
 KVSVTCLIKGFHPPDIAVEWEITGQPEPENNYQTTPQLDSDGTYFLYSRL
 SVDRSHWQRGNTYTCVSVSHEALHSHHTQKSLTQSPG.

4. The recombinant cell of claim 3, wherein the cDNA comprises the following nucleic acid sequence:

(SEQ ID NO: 37)
 atggaatggagctgggtctttctctctctctcagtaacgactggtgtc
 cactcctcgtgaaccagcactgtgcggtccgacctggggaagctctg
 tatctcgtgtgcccgcagcgggctctctacaccgatcccactggagge
 ggtccacgcagagcatcgtggaacagtgtgccactccatctgctccctg
 taccagctggaaaactactgcaatggcggaggtggttcaggaggcgtgga
 ggtgagggcccaagtgcccggtgccagatcccggtgccccagcgtg
 ttcattcttccccaaaacccaaggacaccctgagcatcagcaggaccccc
 gaggtgacctgctggtggtggacctgggacccgacgacgacaacgtgacg
 atcactggttctgtggacaacaccgagatgcacaccgccaagaccaggct
 cgggaggagcagttcaagagcacctacaggggtggtgagcgtgctgccatc

-continued

ctgcaccaggactggctgaagggcaaggagttcaagtgaaggtgaacagc
 aagagcctgccccagcgcctatggagaggaccatcagcaaggccaagggtcag
 ccccacgagccccaagtgtactgtcttccccgaccaggaggattgagc
 gagaacaaagtgagcgtgacctgctgatcaagggcttccaccctcccgac
 atcgccgtggagtgaggatcaccggccagctgagccgaaaataaactac
 cagaccaccctccacagctggacagcagcgacacctacttctttacagc
 aggctgtctgtggaccgaagccattggcaaggggcaaacctacacctgc
 agcgtgagccacagagctctgcacagccaccacccagaagtctctgacc
 cagagccccggatag.

5. The recombinant cell of claim 1, wherein said recombinant cell is a stably transfected recombinant cell.

6. A method of preparing a recombinant cell according to claim 1, said method comprising:

transfecting a host cell with a nucleic acid encoding for said fusion protein comprising said insulin polypeptide and said Fc fragment connected by said linker, wherein said fusion protein is expressed in said recombinant cell after said transfecting step.

7. The method of claim 6, further comprising:

culturing said recombinant cell in cell culture media; and harvesting cell culture supernatant from said cell culture media, said cell culture supernatant comprising said expressed fusion protein.

8. The method of claim 7, further comprising:

recovering said fusion protein from said cell culture supernatant.

9. The method of claim 8, wherein said recovering step comprises one or more steps of centrifugation, filtration, or chromatography.

10. The method of claim 6, wherein said transfecting step comprises stable transfection of said host cell.

11. The method of claim 6, wherein said nucleic acid comprises cDNA encoding said fusion protein, said fusion protein comprising the following sequence:

(SEQ ID NO: 38)
 FVNQHLGSDLVEALYLVCGERGFFYDPTGGGPRRGIVEQCCHSIC
 SLYQLENYCNNGGGSGGGGGEGPKCPVPEIPGAPSVFIFPPKPKDLSISRTPVETCLVVDLGPDDSNVQITWFDNTEMHAKTRPREEQFKS
 TYRVVSVLPILHQDWLKGKFEKCKVNSKSLPSAMERTISKAKQPHEPQVYVLPPTQEELSENKVSVTCLIKGFHPPDIAVEWEITGQPEPENNYQTTPQLDSDGTYFLYSRLSVDRSHWQRGNTYTCVSVSHEALHSHHTQKSLTQSPG.

12. The method of claim 11, wherein the cDNA comprises the following nucleic acid sequence:

(SEQ ID NO: 37)
 atggaatggagctgggtctttctctctctcagtaacgactggtgtc
 cactcctcgtgaaccagcactgtgcggtccgacctggggaagctctg
 tatctcgtgtgcccgcagcgggctctctacaccgatcccactggagge

- continued

ggtccacgcagaggcatcgtggaacagtgtgccactccatctgctccctg
 taccagctggaaaactactgcaatggcggaggtggttcaggaggcgggtgga
 ggtgaggggcccaagtgtccccgtgccccagatccccggtgccccagcgtg
 ttcattcttccccaaaacccaaggacaccctgagcatcagcaggaccccc
 gaggtagcctgctggtggtggacctgggacccgacgacgacaacgtgacg
 atcacctggttcgtggacaacaccgagatgcacaccgccaagaccaggcct
 cgggaggagcagttcaagagcacctacagggtggtgagcgtgctgccccatc
 ctgaccaggactggtgaagggaagaggttcaagtgaaggtgaacagc
 aagagcctgcccagcgcctaggagaggaccatcagcaaggccaaggtcag
 ccccacgagcccaagtgtactgcttccccgacccaggaggagttagc
 gagaacaaagtgagcgtgacctgctgatcaagggttccaccctcccagc
 atcgccgtggagtgaggatcacccggcagcctgagccgaaaataactac
 cagaccaccctccacagctggacagcagcgccacctacttctttacagc
 aggtgtctgtggaccgaagccattggcaagggtgcaaacctacacctgc
 agcgtgagccacgaggctctgcacagccaccaccccagaagtctctgacc
 cagagccccggatag.

13. A cDNA encoding a fusion protein comprising the following sequence:

(SEQ ID NO: 36)

FVNQHLCGSDLVEALYLVCGERGFFYTDPTGGGPRRGIVEQCCHSICSLYQ
 LENYCNNGGGSGGGGEGPKCPVPEIPGAPSVFIFPPKPKDTLSISRTPEV
 TCLVVDLGPDDSNVQITWFDNTEMHHTAKTRPREEQFKSTYRVVSVLPILH
 QDWLKGKEFKCKVNSKSLPSAMERTISKAKGQPHQPQVYVLPPTQEELSEN
 KVSVTCLIKGFHPPDIAVEWEITGQPEPENNYQTTPQLDSDGTYFLYSRL
 SVDRSHWQRGNTYTCVSHALSHHTQKSLTQSPG.

14. The cDNA of claim 13, wherein the cDNA comprises the following nucleic acid sequence:

(SEQ ID NO: 37)

atggaatggagctgggtctttctcttctctctgtcagtaaccgactggtgtc
 cactccttcgtgaaccagcacctgtgcggtccgacctggtggaagctctg
 tatctcgtgtgctggcgagcggggcttcttctacaccgatcccactggaggc
 ggtccacgcagaggcatcgtggaacagtgtgccactccatctgctccctg
 taccagctggaaaactactgcaatggcggaggtggttcaggaggcgggtgga
 ggtgaggggcccaagtgtccccgtgccccagatccccggtgccccagcgtg
 ttcattcttccccaaaacccaaggacaccctgagcatcagcaggaccccc
 gaggtagcctgctggtggtggacctgggacccgacgacgacaacgtgacg
 atcacctggttcgtggacaacaccgagatgcacaccgccaagaccaggcct
 cgggaggagcagttcaagagcacctacagggtggtgagcgtgctgccccatc
 ctgaccaggactggctgaagggaagaggttcaagtgaaggtgaacagc
 aagagcctgcccagcgcctaggagaggaccatcagcaaggccaaggtcag
 ccccacgagcccaagtgtactgcttccccgacccaggaggagttagc
 gagaacaaagtgagcgtgacctgctgatcaagggttccaccctcccagc
 atcgccgtggagtgaggatcacccggcagcctgagccggaaaataactac
 cagaccaccctccacagctggacagcagcgccacctacttctttacagc
 aggtgtctgtggaccgaagccattggcaagggtgcaaacctacacctgc
 agcgtgagccacgaggctctgcacagccaccaccccagaagtctctgacc
 cagagccccggatag.

* * * * *