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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 August 2015 (06.08.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/114341 A1

(51) International Patent Classification:
A61K 47/48 (2006.01) *A61P 3/10* (2006.01)

(21) International Application Number:
PCT/GB2015/050210

(22) International Filing Date:
29 January 2015 (29.01.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1401706.5 31 January 2014 (31.01.2014) GB

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

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

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

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

WO 2015/114341 A1

(54) Title: NANOPARTICLE-INSULIN AND INSULIN ANALOGUE COMPOSITIONS

(57) Abstract: The present invention relates to insulin- and insulin analogue-carrying nanoparticles formulated together with or for use with free insulin or with an insulin analogue, such as a rapidly-acting insulin analogue. The compositions of the present invention find use in medicine, particularly in glycaemic control, e.g. for controlling blood glucose levels in diabetic subjects. Nanoparticle compositions of the invention, formulated together with or for use with free insulin or with an insulin analogue, comprise a nanoparticle comprising a core comprising a metal and/or a semiconductor; and a corona comprising a plurality of ligands covalently linked to the core, wherein said plurality of ligands comprise at least one carbohydrate moiety; and at least one insulin peptide and/or insulin analogue peptide that is non-covalently bound to the corona.

Nanoparticle-Insulin and Insulin Analogue Compositions

Field of the invention

The present invention relates to peptide-carrying nanoparticles, particularly for use in medicine, and includes methods for treatment of disorders, e.g., of diabetes.

Background to the invention

The present invention is directed at compositions and products, and methods of making and administering such compositions and products, including for the treatment of mammals and particularly humans.

Bioactive agents, such as peptides, frequently suffer from poor stability, particularly thermo-stability, which may limit the conditions to which the agents can be subjected during preparation, processing, storage and/or delivery. Medical preparations of peptides for human use are generally formulated with one or more preservatives and/or stabilisers. Moreover, limited gastrointestinal stability typically presents a barrier to effective oral administration of bioactive peptides.

WO 2011/154711 describes glyconanoparticles that have a gold core surrounded by a carbohydrate corona and which act as carriers for peptides such as insulin.

Fast-acting analogues of insulin include insulin lispro (Humalog®, Eli Lilly and Company), insulin aspart (NovoRapid®, Novo Nordisk A/S) and insulin glulisine (Apidra®, Sanofi-Aventis). Fast-acting insulin analogues are typically administered as meal time insulin for post-prandial glucose control in diabetic patients. Compared with regular insulin, these analogue exhibit a shortened delay of onset, allowing more flexibility in the coordination of meal times and insulin administration.

There remains an unmet need for further bioactive peptide compositions, in particular for delivery of insulin and rapid-acting

analogues thereof. There remains an unmet need for pharmaceutical compositions and/or formulations of rapid acting insulin analogues which restore the neural counter-hormone response of such analogues and thereby reduce the incidence of hypoglycaemia.

The present invention addresses these and other needs.

Brief Description of the Invention

Broadly, the present invention relates to formulations of insulin-carrying nanoparticles, including co-formulations of one or more rapid-acting insulin analogues with insulin analogue-carrying nanoparticles. The present inventors have found that nanoparticle-carried fast-acting insulin analogues exhibit a hormetic-type response comparable to normal free insulin, a response that is largely lacking from the same fast-acting insulin analogues when administered as free peptides. The restoration of the "lost" neural counter-hormone response is both advantageous and unexpected. It is presently believed, based on the data described herein, that the inclusion of nanoparticle-carried fast-acting insulin analogue in a pharmaceutical formulation of the fast-acting insulin analogue will reduce the likelihood or incidence of unwanted hypoglycaemic events, e.g. among insulin-dependent diabetic subjects.

Accordingly, in a first aspect the present invention provides a pharmaceutical composition comprising:

(i) free insulin or a free peptide analogue thereof; and

(ii) a plurality of nanoparticles, each of said nanoparticles comprising:

(a) a core comprising a metal and/or a semiconductor;

(b) a corona comprising a plurality of ligands covalently linked to the core, wherein at least one of said ligands comprises a carbohydrate moiety; and

(c) at least one insulin molecule or peptide analogue thereof non-covalently bound to the corona,

wherein the molar ratio of free insulin or free peptide analogue thereof to the nanoparticle-bound insulin or nanoparticle-bound peptide analogue thereof is in the range 1:1 to 100:1.

In some cases in accordance with this and other aspects of the present invention, the peptide analogue of insulin has at least 70%, 80%, 90%, 95% or at least 99% amino acid sequence identity to the human insulin sequence of SEQ ID NO: 1 and is of between 40 and 60 amino acids in length.

In some cases in accordance with this and other aspects of the present invention, the peptide analogue of insulin is selected from the group consisting of: insulin glulisine; insulin aspart; insulin lispro; NPH insulin; insulin glargine; insulin detemir; and insulin degludec.

In some cases in accordance with this and other aspects of the present invention, the peptide analogue of insulin exhibits a central nervous system uptake in a mammalian subject that is 50%, 20%, 10%, 5% or less of the a central nervous system uptake of native human insulin having the amino acid sequence of SEQ ID NO: 1.

In some cases in accordance with this and other aspects of the present invention, the peptide analogue of insulin is an insulin analogue that induces at least transient hypoglycaemia when administered to a mammalian subject as a free peptide composition in the absence of nanoparticle-bound insulin or nanoparticle-bound insulin analogue.

In some cases in accordance with this and other aspects of the present invention, the nanoparticle-bound insulin or nanoparticle-bound insulin analogue induces an increase in plasma adrenalin concentration and/or plasma growth hormone concentration upon administration to a mammalian subject.

In a second aspect, the present invention provides a method of regulating blood glucose concentration in a diabetic or pre-diabetic

mammalian subject, said method comprising administering to the subject an effective amount of the pharmaceutical composition in accordance with the first aspect of the invention.

In a third aspect, the present invention provides a method of reducing the incidence of hypoglycemic adverse events in an insulin-dependent diabetic or pre-diabetic mammalian subject, said method comprising:

administering, simultaneously, sequentially or concurrently with a dose of insulin or a peptide analogue thereof, an effective amount of the pharmaceutical composition comprising a plurality of nanoparticles, each of said nanoparticles comprising:

(a) a core comprising a metal and/or a semiconductor;

(b) a corona comprising a plurality of ligands covalently linked to the core, wherein at least one of said ligands comprises a carbohydrate moiety; and

(c) at least one insulin molecule or peptide analogue thereof non-covalently bound to the corona. When administration is sequential the dose of free insulin or peptide analogue thereof and the dose of nanoparticle-bound insulin or analogue thereof may be administered in any order. Moreover, when administration is sequential the dose of free insulin or peptide analogue thereof and the dose of nanoparticle-bound insulin or analogue thereof may, in some cases, be administered between 1 second and 1 hour apart, e.g. between 1 minute and 10 minutes apart, or between 2 minutes and 5 minutes apart.

In a fourth aspect, the present invention provides a pharmaceutical composition in accordance with the first aspect of the invention for use in medicine.

In a fifth aspect, the present invention provides a pharmaceutical composition in accordance with the first aspect of the invention for use in a method in accordance with the second or third aspects of the present invention.

In a sixth aspect, the present invention provides use of a pharmaceutical composition in accordance with the first aspect of the invention for use in the preparation of a medicament for use in a therapeutic method in accordance with the second or third aspects of the present invention.

In accordance with any one of the aspects of the present invention, the corona may comprise one or more carbohydrate ligands, for example one or more monosaccharide ligands, covalently attached to the core via a linker.

In accordance with any one of the aspects of the present invention, the insulin or analogue thereof may comprise or consist of an amino acid A chain having at least 70%, 80%, 90%, 95% or 99% amino acid sequence identity with the full-length amino acid sequence set forth as SEQ ID NO: 1 and an amino acid B chain having at least 70%, 80%, 90%, 95% or 99% amino acid sequence identity with any one of the full-length amino acid sequences set forth as SEQ ID NOS: 2-5, wherein the A and B chains are linked. In some cases, the link between the A and B chains comprises at least one or at least two disulphide bonds. In some cases, the analogue of insulin comprises an A chain that has up to 1, 2, 3, 4 or 5 amino acid changes by substitution, addition and/or deletion as compared with the full-length A chain amino acid sequence set forth in SEQ ID NO: 1. In some cases, the analogue of insulin comprises a B chain that has up to 1, 2, 3, 4 or 5 amino acid changes by substitution, addition and/or deletion as compared with any one of the full-length B chain amino acid sequences set forth in SEQ ID NOS: 2-5.

The human insulin sequence is disclosed at UniProt accession no. P01308, version 186, dated 13 November 2013. Human insulin is a heterodimer of insulin A chain and insulin B chain linked by two disulphide bonds.

The A chain (consisting of residues 90-110 of the 110 amino acid sequence of preproinsulin) has the following sequence:
>sp|P01308|90-110

GIVEQCCTSICSLYQLENYCN (SEQ ID NO: 1)

The B chain (consisting of residues 25-54 of the 110 amino acid sequence of preproinsulin) has the following sequence:
>sp|P01308|25-54
FVNQHLCGSHLVEALYLVCGERGFFYTPKT (SEQ ID NO: 2)

The A and B chains are linked by two disulphide bonds: a first interchain bond between Cys31 of the B chain and Cys 96 of the A chain (numbered according to the preproinsulin sequence) and a second interchain bond between Cys43 of the B chain and Cys109 of the A chain (numbered according to the preproinsulin sequence).

In some cases in accordance with any aspect of the present invention, the analogue of insulin may be a fast-acting insulin analogue. In particular, the insulin analogue may be selected from the group consisting of: insulin lispro (Humalog®, Eli Lilly and Company), insulin aspart (NovoRapid®, Novo Nordisk A/S) and insulin glulisine (Apidra®, Sanofi-Aventis).

Insulin lispro is a fast-acting insulin analogue having an inversion of Lys28 and Pro29 of the B chain compared with native insulin (i.e. positions 52 and 53 numbered according to the preproinsulin sequence). The B chain of insulin lispro therefore has the amino acid sequence FVNQHLCGSHLVEALYLVCGERGFFYT**KP**T (SEQ ID NO: 3).

Insulin aspart is a fast-acting insulin analogue having the amino acid substitution Pro28Asp in the B chain compared with native insulin (i.e. position 52 numbered according to the preproinsulin sequence). The B chain of insulin aspart therefore has the amino acid sequence FVNQHLCGSHLVEALYLVCGERGFFYT**D**KT (SEQ ID NO: 4).

Insulin glulisine is a fast-acting insulin analogue having the amino acid substitutions Asn3Lys and Lys29Glu in the B chain compared with native insulin (i.e. positions 27 and 53 numbered according to the preproinsulin sequence). The B chain of insulin glulisine therefore

has the amino acid sequence FV**K**QHLCGSHLVEALYLVCGERGFFYTP**E**T (SEQ ID NO: 5).

It has been found that the nanoparticles in accordance with the present invention may be provided with a variety of numbers of ligands forming the corona. For example, in some cases the corona comprises at least 5, 10, 20 or at least 50 ligands per core, e.g. between about 10 to about 1000 ligands per core. In particular, the nanoparticle compositions in accordance with any aspect of the present invention may comprise at least 5, 10, 15, 20 or at least 50 glutathione ligands and/or carbohydrate ligands per core.

The number of insulin peptide molecules and/or insulin analogue peptide molecules bound per core is not particularly limited. For certain applications, it may be desirable to employ as few as 1, 2, 3 or 4 insulin peptides and/or insulin analogue peptides per core, while in other cases the nanoparticle of the invention may comprise at least 5, 10, 15, 20, 30 or at least 50 or more insulin peptides and/or insulin analogue peptides bound per core. In some cases the nanoparticle of the invention may comprise between 10 and 30 (e.g. approximately 20) glulisine peptide molecules per nanoparticle core.

In some cases, in accordance with any one of the aspects of the present invention, the at least one insulin peptide and/or insulin analogue peptide may be bound to the corona of the nanoparticle in a reversible manner. In particular, the insulin peptide and/or insulin analogue peptide may be bound to the corona such that at least a fraction of the bound peptide is released from the nanoparticle upon contacting the nanoparticle with a physiological solution. The insulin peptide and/or insulin analogue peptide may in some cases be adsorbed to the corona of the nanoparticle. The insulin peptide and/or insulin analogue peptide may in some cases be electrostatically or otherwise non-covalently bound to the one or more ligands that form the corona of the nanoparticle.

In some cases, in accordance with any one of the aspects of the present invention, said ligands comprise a carbohydrate-containing

ligands alone or in conjunction with other species of ligand, e.g., combinations of carbohydrate ligands (including, e.g., alpha-galactose-containing ligands) and non-carbohydrate containing ligands are specifically contemplated herein. The one or more species of ligand may be selected from one or more of the ligands that form the corona of the nanoparticles disclosed in WO2011/154711, the entire contents of which is expressly incorporated herein by reference.

In some cases in accordance with any one of the aspects of the present invention said carbohydrate moiety may comprises a monosaccharide and/or a disaccharide. The carbohydrate moiety may be as defined further herein, including a carbohydrate mimetic. The carbohydrate moiety may be covalently linked to the core via a linker selected from the group consisting of: sulphur-containing linkers, amino-containing linkers, phosphate-containing linkers and oxygen-containing linkers. In some cases the linker comprises an alkyl chain of at least two carbons. In some cases the carbohydrate-containing ligand or ligands may be other than chitosan.

In accordance with the present invention said at least one ligand comprising a carbohydrate moiety may in some cases be selected from the group consisting of: 2´-thioethyl-α-D-galactopyranoside, 2´-thioethyl-β-D-glucopyranoside, 2´-thioethyl-2-acetamido-2-deoxy-β-D-glucopyranoside, 5´-thiopentanyl-2-deoxy-2-imidazolacetamido-α,β-D-glucopyranoside and 2´-thioethyl-α-D-glucopyranoside, wherein said at least one ligand comprising a carbohydrate moiety is covalently linked to the core via its sulphur atom.

It is specifically contemplated herein that said plurality of ligands covalently linked to the core may comprise at least a first ligand and a second ligand, wherein the first and second ligands are different. For example the first and second ligands may be as follows:

(a) said first ligand comprises 2´-thioethyl-α-D-galactopyranoside and said second ligand comprises 1-amino-17-mercapto-3,6,9,12,15,-pentaoxa-heptadecanol;

(b) said first ligand comprises 2´-thioethyl-β-D-glucopyranoside or 2´-thioethyl-α-D-glucopyranoside and said second ligand comprises 5´-thiopentanyl-2-deoxy-2-imidazolacetamido-α,β-D-glucopyranoside;

(c) said first ligand comprises 2´-thioethyl-β-D-glucopyranoside or 2´-thioethyl-α-D-glucopyranoside and said second ligand comprises 1-amino-17-mercapto-3,6,9,12,15,-pentaoxa-heptadecanol; or

(d) said first ligand comprises 2´-thioethyl-2-acetamido-2-deoxy-β-D-glucopyranoside and said second ligand comprises 1-amino-17-mercapto-3,6,9,12,15,-pentaoxa-heptadecanol,

and wherein said first and second ligands are covalently linked to the core via their respective sulphur atoms.

In some cases the first ligand may comprise a carbohydrate moiety and said second ligand a non-carbohydrate ligand. One or more of the ligands may an amine group. In particular, the second ligand may comprise 1-amino-17-mercapto-3,6,9,12,15,-pentaoxa-heptadecanol covalently linked to the core via its sulphur atom.

As described further herein, where there different ligands are present on the nanoparticle they may be present at, e.g., certain defined ratios or ranges of ratios. For example, the first ligand and said second ligand may present on the nanoparticle in a molar ratio in the range of 1:40 to 40:1, 1:10 to 10:1 or even 1:2 to 2:1.

In accordance with the present invention the nanoparticle of the invention may comprise a component having a divalent state, such as a metal or a compound having a divalent state, or an oxide or salt thereof. For example, metals or metal complexes having the ability

to exist in a divalent state are particularly useful. Such a component may be in the divalent state as added or may be transformed into a divalent state after addition. Oxides and salts of the divalent component are also useful and may be added directly or formed in situ subsequent to addition. Among the useful salts of the divalent component include halide salts, such as chloride, iodide, bromide and fluoride. Such divalent components may include, for example, zinc, magnesium, copper, nickel, cobalt, cadmium, or calcium, and their oxides and salts thereof. The component is desirably present in an amount sufficient to produce a stabilizing effect and/or in an amount sufficient to enhance the binding of the peptide to the corona to a level greater than the level of binding of the peptide to the corona in the absence of the component having a divalent state. In some cases, the component having a divalent state is desirably present in an amount of about 0.5 to 2.0 equivalents to the core metal (e.g. gold), or optionally about 0.75 to 1.5 equivalents to the core metal (e.g. gold). In the context of the present invention, "equivalents" may be mole equivalents, for example 1.0 equivalent of zinc may be taken to mean the same number of zinc atoms or Zn^{2+} cations as the number of gold atoms in the core of the nanoparticle.

The divalent component may in some cases be present in the corona of the nanoparticle. It is specifically contemplated herein that the divalent component may be included in the nanoparticle, including in the corona of the nanoparticle as a result of inclusion of the divalent component in the process of synthesis of the nanoparticle. Additionally or alternatively, the divalent component may be added after synthesis of the nanoparticle. In some cases in accordance with the present invention, the divalent component, such as zinc may be selected from: Zn^{2+} and ZnO. For example, the zinc may be in the form of $ZnCl_2$.

In some cases, in accordance with any one of the aspects of the present invention, the diameter of the core of the nanoparticle is in the range 1 nm to 5 nm.

In some cases, in accordance with any one of the aspects of the present invention, the diameter of the nanoparticle including its ligands is in the range 2 nm to 50 nm, optionally 3 nm to 30 nm, or 4 nm to 20 nm, or 5 nm to 15 nm.

In some cases, in accordance with any one of the aspects of the present invention, the core comprises a metal selected from the group consisting of: Au, Ag, Cu, Pt, Pd, Fe, Co, Gd and Zn, or any combination thereof.

In some cases, in accordance with any one of the aspects of the present invention, the core is magnetic.

In some cases, in accordance with any one of the aspects of the present invention, the core comprises a semiconductor. The semiconductor may comprise metal atoms, such as cadmium. Alternatively or additionally, the semiconductor may comprise non-metal atoms. Organic semiconductors are specifically contemplated herein. Preferred semiconductors, in accordance with the present invention, may be selected from the group consisting of: cadmium selenide, cadmium sulphide, cadmium tellurium and zinc sulphide.

In some cases, in accordance with any one of the aspects of the present invention, the core is capable of acting as a quantum dot.

Preferably, the composition in accordance with the first aspect of the invention comprises a plurality, e.g., 100, 1000, 100000, or more, of said nanoparticles, wherein at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% of the nanoparticles in said composition have at least one insulin or insulin analogue peptide bound.

In some cases, in accordance with any one of the aspects of the present invention, the plurality of nanoparticles are formulated in a carrier, such as solution, a polymer (e.g. a viscoelastic polymer film), a powder, or a cream, in which the nanoparticles and bound insulin and/or insulin analogue peptides are suspended and/or

embedded. In certain cases, the nanoparticle formulation may be in the form of a patch or film for delivery to or across skin, mouth, cheek (transbuccal), vagina, rectum or in the form of a spray for delivery into the mouth, nose, lungs or the rectum or vagina. The formulation may be in an associated form, a suspension or contained together in a single package, container or carrier (e.g. wherein both the plurality of nanoparticles and the free peptide insulin or free peptide insulin analogue are in a single package, container or carrier).

In certain cases, the pharmaceutical composition in accordance with any one of the aspects of the present invention may take the form of one or more doses (e.g. a defined quantity of insulin peptide or analogue thereof, or insulin peptide activity units), such as in the form of a therapeutic dose or defined number of doses. In certain cases the nanoparticle portion of the pharmaceutical composition may be in the form of a viscoelastic polymer film for transbuccal delivery.

In some cases, in accordance with any one of the aspects of the present invention, the plurality of nanoparticles may further comprise at least one permeation enhancer that is non-covalently or covalently bound to said core and/or or said corona. As described in co-pending GB patent application No. 1301991.4, filed 5 February 2013, the entire contents of which are expressly incorporated herein by reference for all purposes, certain permeation enhancers may be advantageously bound to the nanoparticle without displacing any significant active peptide, such as the insulin peptide or analogue thereof as defined herein. In certain cases, said permeation enhancer is selected from tetradecyl-D-maltoside and lysalbinic acid. In certain cases, said permeation enhancer, e.g. tetradecyl-D-maltoside and/or lysalbinic acid is non-covalently bound to said corona.

In accordance with any one of the second to sixth aspects of the invention, the subject may be a human, a companion animal (e.g. a dog or cat), a laboratory animal (e.g. a mouse, rat, rabbit, pig or

non-human primate), a domestic or farm animal (e.g. a pig, cow, horse or sheep). Preferably, the subject is a human. In some cases the subject has type 1 diabetes, type 2 diabetes or prediabetes.

In accordance with any one of the second to sixth aspects of the invention, the subject may in certain cases have a disorder that results in abnormally lowered blood glucose concentration (i.e. hypoglycaemia). Hypoglycaemia may be a temporary state resulting from poor management of a diabetic condition, for example where too much insulin has been administered or insufficient food taken in or the insulin and food intake have been poorly timed such that the subject enters a state of hypoglycaemia. Without wishing to be bound by any particular theory, the present inventors believe that the comparative paucity of neural recognition of rapidly-acting insulin analogues when delivered in conventional formulations may result in unwanted hypoglycaemia (e.g. hypoglycaemia may result if a rapidly-acting insulin analogue is administered and the subject does not then eat within around 15 minutes). However, as described in the examples herein, insulin or analogues thereof (including rapidly-acting analogues) delivered bound to nanoparticles as defined herein are believed to exhibit superior neural recognition in a mammalian subject as compared with rapidly-acting insulin analogues delivered in conventional formulations (e.g. via subcutaneous injection of free peptide formulations). Therefore, nanoparticle-bound insulin and/or insulin analogue peptide may be advantageously administered with a rapidly-acting insulin analogue at or around the time that the rapidly-acting insulin analogue is administered in a conventional formulation (e.g. via subcutaneous injection of free peptide formulations) and thereby reducing or avoiding hypoglycaemia induced by the free rapidly-acting insulin analogue peptide.

In accordance with any one of the second to sixth aspects of the invention, it is specifically contemplated that, in some cases, the composition of the invention may be self-administered or for self-administration.

In accordance with any one of the second to sixth aspects of the invention, the plurality of nanoparticles may be administered or for administration with (i.e. simultaneously, separately or sequentially) one or more therapeutic agents for the control of diabetes.

In accordance with any one of the second to sixth aspects of the invention, the plurality of nanoparticles may be administered or for administration by any suitable route. In particular cases, the plurality of nanoparticles may be administered or for administration via a route selected from the group consisting of: intravenous (i.v.), intramuscular (i.m.), intradermal (i.d.), intraperitoneal or subcutaneous (s.c.) injection or infusion; buccal; sublabial; sublingual; by inhalation; via one or more mucosal membranes; urogenital; rectal; intranasal and dermal.

In a seventh aspect, the present invention provides an article of manufacture comprising:

a pharmaceutical composition as defined in accordance with the first aspect of the invention;

a container for housing the pharmaceutical composition; and

an insert and/or label. Preferably, the insert and/or label provide instructions, dosage and/or administration information relating to the use of the pharmaceutical composition in a method of managing blood glucose concentration (glycaemic control) and/or a method of managing or treating diabetes.

In an eighth aspect, the present invention provides a process for producing a pharmaceutical composition as defined in accordance with the first aspect of the invention, the process comprising:

providing a nanoparticle comprising a core comprising a metal and/or a semiconductor and a corona comprising a plurality of ligands covalently linked to the core, wherein said ligands comprise one or more carbohydrate-containing ligands (e.g. galactose-containing ligands);

contacting the nanoparticle with at least one insulin peptide and/or insulin analogue peptide as defined herein under conditions

which allow the at least one insulin peptide and/or insulin analogue peptide peptide to bind to the corona of the nanoparticle; and

combining the resulting nanoparticle having insulin peptide and/or insulin analogue peptide bound thereto with insulin peptide and/or insulin analogue peptide that is not bound to a nanoparticle.

In some cases in accordance with this aspect of the present invention, said combining step comprises including the nanoparticle having insulin peptide and/or insulin analogue peptide bound thereto in a housing or container with the insulin peptide and/or insulin analogue peptide that is not bound to a nanoparticle. The insulin analogue peptide may be a rapidly-acting insulin analogue as further defined herein.

In some cases, in accordance with this aspect of the present invention, the process comprises an earlier step of producing the nanoparticle, said earlier step comprising: combining a solution comprising one or more derivatised carbohydrate moieties (e.g. thioethyl-alpha galactose) with a solution comprising a core-forming material (e.g. gold III chloride) and with a reducing agent (e.g. sodium borohydride), thereby causing the nanoparticle to self-assemble.

The present invention includes the combination of the aspects and preferred features described except where such a combination is clearly impermissible or is stated to be expressly avoided. These and further aspects and embodiments of the invention are described in further detail below and with reference to the accompanying examples and figures.

Brief Description of the figures

Figure 1 shows minipig plasma glucose plotted against time for 1.2 U/kg gold nanoparticle-bound insulin ("GNP-I"; filled squares); 0.3 U/kg GNPI (filled triangles); and 1.2 U/kg free insulin lispro (filled circles). Somatostatin is indicated by the dark bar at the top of the figure. The results indicate that for equal subcutaneous

(s.c.) dose of analogue insulin lispro and GNP-regular insulin the analogue insulin lispr results in hypoglycaemia relative to nanoparticle-bound insulin.

Figure 2 shows blood glucose clearance rates in minipigs. **A**) Control animals receiving no insulin; **B**) Test animals receiving intravenous (i.v.) nanoparticle-bound insulin; **C**) Control animals receiving 2.5 units of subcutaneous (s.c.) NovoRapid® insulin aspart; **D**) Test animals receiving transbuccal GNP-regular insulin formulated in PharmFilm™. Each of these curves can be transformed, and early (K1), and late (K2) rate constants for glucose clearance calculated. The results show hypoglycemia for s.c. NovoRapid® (see panel C), but no hypoglycemia for nanoparticle-bound insulin (see panels B and D).

Figure 3 shows the effect of subcutaneous (s.c.) injection of insulin in an insulin stress test. **A**) An s.c. injection of 2.5 U/animal regular (Diosynth) free insulin was administered to anaesthetized minipigs (1-squares; 2-circles; 3-triangles; and 4-inverted triangles) and blood glucose concentration plotted against time. **B**) An s.c. injection of 2.5 U/animal gold nanoparticle (GNP)-bound regular (Diosynth) insulin was administered to anaesthetized minipigs (1-squares; 2-circles; 3-triangles; and 4-inverted triangles) and blood glucose concentration plotted against time. **C**) In a further experiment, an s.c. injection of 2.5 U/animal gold nanoparticle (GNP)-bound regular (Diosynth) insulin was administered to anaesthetized minipigs (1-squares; 2-circles; 3-triangles; and 4-inverted triangles) and blood glucose concentration plotted against time. The results show that in these insulin stress tests neither s.c. administration of regular insulin or GNP-I insulin results in hypoglycaemia.

Figure 4 shows an insulin stress test following s.c. injection of 0.15 IU/kg free Apidra® (analogue insulin glulisine; circles; n=8) or transbuccal delivery of gold nanoparticle (GNP)-bound Apidra® (analogue insulin glulisine; squares; n=8) in anesthetized minipigs. Blood glucose concentration is plotted against time. Significant features of the glucose response patterns are indicated. These

results show that s.c. administration of the rapidly-acting insulin analogue Apidra® results in hypoglycaemia, whereas transbuccal administration of GNP-bound Apidra® does not exhibit hypoglycaemia.

Figure 5 shows that s.c. Apidra® does not induce counter-hormones during anaesthesia. **A**) Adrenalin (left-hand y-axis) and glucose (right-hand y-axis) concentrations are plotted against time for 0.15 U/kg s.c. administered Apidra® in anaesthetised minipigs. **B**) Glucagon (left-hand y-axis) and glucose (right-hand y-axis) concentrations are plotted against time for 0.15 U/kg s.c. administered Apidra® in anaesthetised minipigs. **C**) Growth hormone (left-hand y-axis) and glucose (right-hand y-axis) concentrations are plotted against time for 0.15 U/kg s.c. administered Apidra® in anaesthetised minipigs. The results shown in this insulin stress test demonstrate that, despite induction of hypoglycaemia, the Apidra® does not generate a counter-hormone response. S.c. administered Apidra® only induces counter-hormone release post-anesthesia; there is no induction of early hypoglycaemia recognition.

Figure 6 shows that transbuccally administered gold nanoparticle-bound Apridra® triggers a neural counter-hormone response in an insulin stress test in minipigs. **A**) Adrenalin (left-hand y-axis) and glucose (right-hand y-axis) concentrations are plotted against time for transbuccally administered, gold nanoparticle-bound Apidra® (TB-GNP-Apidra®). **B**) Growth hormone (GH) (left-hand y-axis) and glucose (right-hand y-axis) concentrations are plotted against time for transbuccally administered, gold nanoparticle-bound Apidra® (TB-GNP-Apidra®). The observed immediate rise of plasma adrenalin and GH to TB-GNP-Apidra® could explain the quick rise in blood glucose post-TB-GNP-Apidra® administration.

Detailed description of the invention

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

As used herein, "nanoparticle" refers to a particle having a nanomeric scale, and is not intended to convey any specific shape limitation. In particular, "nanoparticle" encompasses nanospheres, nanotubes, nanoboxes, nanoclusters, nanorods and the like. In certain embodiments the nanoparticles and/or nanoparticle cores contemplated herein have a generally polyhedral or spherical geometry.

Nanoparticles comprising a plurality of carbohydrate-containing ligands have been described in, for example, WO 2002/032404, WO 2004/108165, WO 2005/116226, WO 2006/037979, WO 2007/015105, WO 2007/122388, WO 2005/091704, WO 2011/154711 (the entire contents of each of which is expressly incorporated herein by reference) and such nanoparticles may find use in accordance with the present invention. Moreover, gold-coated nanoparticles comprising a magnetic core of iron oxide ferrites (having the formula XFe_2O_4, where X = Fe, Mn or Co) functionalised with organic compounds (e.g. via a thiol-gold bond) are described in EP2305310 (the entire contents of which is expressly incorporated herein by reference) and are specifically contemplated for use as nanoparticles/nanoparticle cores in accordance with the present invention.

As used herein, "corona" refers to a layer or coating, which may partially or completely cover the exposed surface of the nanoparticle core. The corona includes a plurality of ligands which generally include at least one carbohydrate moiety, one surfactant moiety and/or one glutathione moiety. Thus, the corona may be considered to be an organic layer that surrounds or partially surrounds the metallic and/or semiconductor core. In certain embodiments the corona provides and/or participates in "passivating" the core of the nanoparticle. Thus, in certain cases the corona may include a sufficiently complete coating layer substantially to stabilise the semiconductor or metal-containing core. However, it is specifically contemplated herein that certain nanoparticles having cores, e.g., that include a metal oxide-containing inner core coated with a noble metal may include a corona that only partially coats the core surface. In certain cases the corona facilitates

solubility, such as water solubility, of the nanoparticles of the present invention.

Nanoparticles

Nanoparticles are small particles, e.g. clusters of metal or semiconductor atoms, that can be used as a substrate for immobilising ligands.

Preferably, the nanoparticles have cores having mean diameters between 0.5 and 50nm, more preferably between 0.5 and 10nm, more preferably between 0.5 and 5nm, more preferably between 0.5 and 3nm and still more preferably between 0.5 and 2.5nm. When the ligands are considered in addition to the cores, preferably the overall mean diameter of the particles is between 2.0 and 20 nm, more preferably between 3 and 10 nm and most preferably between 4 and 5 nm. The mean diameter can be measured using techniques well known in the art such as transmission electron microscopy.

The core material can be a metal and/or semiconductor (said semiconductor optionally comprising metal atoms or being an organic semiconductor) and may be formed of more than one type of atom. Preferably, the core material is a metal selected from Au, Fe or Cu. Nanoparticle cores may also be formed from alloys including Au/Fe, Au/Cu, Au/Gd, Au/Fe/Cu, Au/Fe/Gd and Au/Fe/Cu/Gd, and may be used in the present invention. Preferred core materials are Au and Fe, with the most preferred material being Au. The cores of the nanoparticles preferably comprise between about 100 and 500 atoms (e.g. gold atoms) to provide core diameters in the nanometre range. Other particularly useful core materials are doped with one or more atoms that are NMR active, allowing the nanoparticles to be detected using NMR, both *in vitro* and *in vivo*. Examples of NMR active atoms include Mn^{+2}, Gd^{+3}, Eu^{+2}, Cu^{+2}, V^{+2}, Co^{+2}, Ni^{+2}, Fe^{+2}, Fe^{+3} and lanthanides^{+3}, or the quantum dots described elsewhere in this application.

Nanoparticle cores comprising semiconductor compounds can be detected as nanometre scale semiconductor crystals are capable of

acting as quantum dots, that is they can absorb light thereby exciting electrons in the materials to higher energy levels, subsequently releasing photons of light at frequencies characteristic of the material. An example of a semiconductor core material is cadmium selenide, cadmium sulphide, cadmium tellurium. Also included are the zinc compounds such as zinc sulphide.

In some embodiments, the core of the nanoparticles may be magnetic and comprise magnetic metal atoms, optionally in combination with passive metal atoms. By way of example, the passive metal may be gold, platinum, silver or copper, and the magnetic metal may be iron or gadolinium. In preferred embodiments, the passive metal is gold and the magnetic metal is iron. In this case, conveniently the ratio of passive metal atoms to magnetic metal atoms in the core is between about 5:0.1 and about 2:5. More preferably, the ratio is between about 5:0.1 and about 5:1. As used herein, the term "passive metals" refers to metals which do not show magnetic properties and are chemically stable to oxidation. The passive metals may be diamagnetic or superparamagnetic. Preferably, such nanoparticles are superparamagnetic.

Examples of nanoparticles which have cores comprising a paramagnetic metal, include those comprising Mn^{+2}, Gd^{+3}, Eu^{+2}, Cu^{+2}, V^{+2}, Co^{+2}, Ni^{+2}, Fe^{+2}, Fe^{+3} and lanthanides^{+3}.

Other magnetic nanoparticles may be formed from materials such as MnFe (spinel ferrite) or CoFe (cobalt ferrite) can be formed into nanoparticles (magnetic fluid, with or without the addition of a further core material as defined above. Examples of the self-assembly attachment chemistry for producing such nanoparticles is given in Biotechnol. Prog., 19:1095-100 (2003), J. Am. Chem. Soc. 125:9828-33 (2003), J. Colloid Interface Sci. 255:293-8 (2002).

In some embodiments, the nanoparticle or its ligand comprises a detectable label. The label may be an element of the core of the nanoparticle or the ligand. The label may be detectable because of an intrinsic property of that element of the nanoparticle or by

being linked, conjugated or associated with a further moiety that is detectable. Preferred examples of labels include a label which is a fluorescent group, a radionuclide, a magnetic label or a dye. Fluorescent groups include fluorescein, rhodamine or tetramethyl rhodamine, Texas-Red, Cy3, Cy5, etc., and may be detected by excitation of the fluorescent label and detection of the emitted light using Raman scattering spectroscopy (Y.C. Cao, R. Jin, C. A. Mirkin, Science 2002, 297: 1536-1539).

In some embodiments, the nanoparticles may comprise a radionuclide for use in detecting the nanoparticle using the radioactivity emitted by the radionuclide, e.g. by using PET, SPECT, or for therapy, i.e. for killing target cells. Examples of radionuclides commonly used in the art that could be readily adapted for use in the present invention include ^{99m}Tc, which exists in a variety of oxidation states although the most stable is TcO^{4-}; ^{32}P or ^{33}P; ^{57}Co; ^{59}Fe; ^{67}Cu which is often used as Cu^{2+} salts; ^{67}Ga which is commonly used a Ga^{3+} salt, e.g. gallium citrate; ^{68}Ge; ^{82}Sr; ^{99}Mo; ^{103}Pd; ^{111}In which is generally used as In^{3+} salts; ^{125}I or ^{131}I which is generally used as sodium iodide; ^{137}Cs; ^{153}Gd; ^{153}Sm; ^{158}Au; ^{186}Re; ^{201}Tl generally used as a Tl^{+} salt such as thallium chloride; $^{39}Y^{3+}$; $^{71}Lu^{3+}$; and $^{24}Cr^{2+}$. The general use of radionuclides as labels and tracers is well known in the art and could readily be adapted by the skilled person for use in the aspects of the present invention. The radionuclides may be employed most easily by doping the cores of the nanoparticles or including them as labels present as part of ligands immobilised on the nanoparticles.

Additionally or alternatively, the nanoparticles of the present invention, or the results of their interactions with other species, can be detected using a number of techniques well known in the art using a label associated with the nanoparticle as indicated above or by employing a property of them. These methods of detecting nanoparticles can range from detecting the aggregation that results when the nanoparticles bind to another species, e.g. by simple visual inspection or by using light scattering (transmittance of a solution containing the nanoparticles), to using sophisticated

techniques such as transmission electron microscopy (TEM) or atomic force microscopy (AFM) to visualise the nanoparticles. A further method of detecting metal particles is to employ plasmon resonance that is the excitation of electrons at the surface of a metal, usually caused by optical radiation. The phenomenon of surface plasmon resonance (SPR) exists at the interface of a metal (such as Ag or Au) and a dielectric material such as air or water. As changes in SPR occur as analytes bind to the ligand immobilised on the surface of a nanoparticle changing the refractive index of the interface. A further advantage of SPR is that it can be used to monitor real time interactions. As mentioned above, if the nanoparticles include or are doped with atoms which are NMR active, then this technique can be used to detect the particles, both *in vitro* or *in vivo*, using techniques well known in the art. Nanoparticles can also be detected using a system based on quantitative signal amplification using the nanoparticle-promoted reduction of silver (I). Fluorescence spectroscopy can be used if the nanoparticles include ligands as fluorescent probes. Also, isotopic labelling of the carbohydrate can be used to facilitate their detection.

Insulin peptide analogues

In certain cases in accordance with the present invention, the "insulin analogue" may be any derivative of, mutant form of, or synthetic mimic of, insulin. In particular the insulin analogue may comprise or consist of an amino acid A chain having at least 70%, 80%, 90%, 95% or 99% amino acid sequence identity with the full-length amino acid sequence set forth as SEQ ID NO: 1 and an amino acid B chain having at least 70%, 80%, 90%, 95% or 99% amino acid sequence identity with any one of the full-length amino acid sequences set forth as SEQ ID NOS: 2-5, wherein the A and B chains are linked. In some cases, the link between the A and B chains comprises at least one or at least two disulphide bonds. In some cases, the analogue of insulin comprises an A chain that has up to 1, 2, 3, 4 or 5 amino acid changes by substitution, addition and/or deletion as compared with the full-length A chain amino acid

sequence set forth in SEQ ID NO: 1. In some cases, the analogue of insulin comprises a B chain that has up to 1, 2, 3, 4 or 5 amino acid changes by substitution, addition and/or deletion as compared with any one of the full-length B chain amino acid sequences set forth in SEQ ID NOS: 2-5.

The human insulin sequence is disclosed at UniProt accession no. P01308, version 186, dated 13 November 2013. Human insulin is a heterodimer of insulin A chain and insulin B chain linked by two disulphide bonds.

The A chain (consisting of residues 90-110 of the 110 amino acid sequence of preproinsulin) has the following sequence:
>sp|P01308|90-110
GIVEQCCTSICSLYQLENYCN (SEQ ID NO: 1)

The B chain (consisting of residues 25-54 of the 110 amino acid sequence of preproinsulin) has the following sequence:
>sp|P01308|25-54
FVNQHLCGSHLVEALYLVCGERGFFYTPKT (SEQ ID NO: 2)

The A and B chains are linked by two disulphide bonds: a first interchain bond between Cys31 of the B chain and Cys 96 of the A chain (numbered according to the preproinsulin sequence) and a second interchain bond between Cys43 of the B chain and Cys109 of the A chain (numbered according to the preproinsulin sequence).

In some cases in accordance with any aspect of the present invention, the analogue of insulin may be a fast-acting insulin analogue. In particular, the insulin analogue may be selected from the group consisting of: insulin lispro (Humalog®, Eli Lilly and Company), insulin aspart (NovoRapid®, Novo Nordisk A/S) and insulin glulisine (Apidra®, Sanofi-Aventis).

Insulin lispro is a fast-acting insulin analogue having an inversion of Lys28 and Pro29 of the B chain compared with native insulin (i.e. positions 52 and 53 numbered according to the preproinsulin

sequence). The B chain of insulin lispro therefore has the amino acid sequence FVNQHLCGSHLVEALYLVCGERGFFYT**KP**T (SEQ ID NO: 3).

Insulin aspart is a fast-acting insulin analogue having the amino acid substitution Pro28Asp in the B chain compared with native insulin (i.e. position 52 numbered according to the preproinsulin sequence). The B chain of insulin aspart therefore has the amino acid sequence FVNQHLCGSHLVEALYLVCGERGFFYT**D**KT (SEQ ID NO: 4).

Insulin glulisine is a fast-acting insulin analogue having the amino acid substitutions Asn3Lys and Lys29Glu in the B chain compared with native insulin (i.e. positions 27 and 53 numbered according to the preproinsulin sequence). The B chain of insulin glulisine therefore has the amino acid sequence FV**K**QHLCGSHLVEALYLVCGERGFFYTP**E**T (SEQ ID NO: 5).

Sequence identity may be calculated using any suitable method, as would be readily apparent to the skilled person. In certain cases, amino acid sequence identity between a candidate sequence and a reference sequence, e.g. the sequence of SEQ ID NO: 1, may be calculated using the online tool SUPERMATCHER available at the following URL: http://emboss.bioinformatics.nl/cgi-bin/emboss/supermatcher using GAP opening penalty of 10.0 and GAP extension penalty of 0.5 (see EMBOSS: The European Molecular Biology Open Software Suite (2000) Rice, P. Longden, I. and Bleasby, A. *Trends in Genetics* 16, (6) pp.276-277).

Administration and treatment

The nanoparticles and compositions of the invention may be administered to patients by any number of different routes, including enteral or parenteral routes. Parenteral administration includes administration by the following routes: intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraocular, transepithelial, intraperitoneal and topical (including dermal, ocular, rectal, nasal, inhalation and aerosol), film, patch and rectal systemic routes. In some cases the nanoparticles and

compositions of the invention may be administered or for administration via transbuccal route.

Administration be performed e.g. by injection, or ballistically using a delivery gun to accelerate their transdermal passage through the outer layer of the epidermis. The nanoparticles may also be delivered in aerosols. This is made possible by the small size of the nanoparticles.

The nanoparticles of the invention may be formulated as pharmaceutical compositions that may be in the forms of solid or liquid compositions. In some cases the nanoparticles may be formulated in a viscoelastic film, e.g. for transbuccal delivery. Compositions in accordance with the present invention will generally comprise a carrier of some sort, for example a solid carrier or a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included. Such compositions and preparations generally contain at least 0.1 wt% of the compound.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, solutions of the compounds or a derivative thereof, e.g. in physiological saline, a dispersion prepared with glycerol, liquid polyethylene glycol or oils.

In addition to one or more of the compounds, optionally in combination with other active ingredient, the compositions can comprise one or more of a pharmaceutically acceptable excipient, carrier, buffer, stabiliser, isotonicising agent, preservative or anti-oxidant or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of

the carrier or other material may depend on the route of administration, e.g. intravenously, orally or parenterally.

Liquid pharmaceutical compositions are typically formulated to have a pH between about 3.0 and 9.0, more preferably between about 4.5 and 8.5 and still more preferably between about 5.0 and 8.0. The pH of a composition can be maintained by the use of a buffer such as acetate, citrate, phosphate, succinate, Tris or histidine, typically employed in the range from about 1 mM to 50 mM. The pH of compositions can otherwise be adjusted by using physiologically acceptable acids or bases.

Preservatives are generally included in pharmaceutical compositions to retard microbial growth, extending the shelf life of the compositions and allowing multiple use packaging. Examples of preservatives include phenol, meta-cresol, benzyl alcohol, para-hydroxybenzoic acid and its esters, methyl paraben, propyl paraben, benzalconium chloride and benzethonium chloride. Preservatives are typically employed in the range of about 0.1 to 1.0 % (w/v).

Preferably, the pharmaceutically compositions are given to an individual in a prophylactically effective amount or a therapeutically effective amount (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. Typically, this will be to cause a therapeutically useful activity providing benefit to the individual. The actual amount of the compounds administered, and rate and time-course of administration, will depend on the nature and severity of the condition being treated. Prescription of treatment, e.g. decisions on dosage etc., is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Handbook of Pharmaceutical Additives, 2nd Edition (eds. M. Ash and I. Ash), 2001 (Synapse Information Resources, Inc., Endicott, New York, USA);

Remington's Pharmaceutical Sciences, 20th Edition, 2000, pub. Lippincott, Williams & Wilkins; and Handbook of Pharmaceutical Excipients, 2nd edition, 1994. By way of example, and the compositions are preferably administered to patients in dosages of between about 0.01 and 100mg of active compound per kg of body weight, and more preferably between about 0.5 and 10mg/kg of body weight.

The following is presented by way of example and is not to be construed as a limitation to the scope of the claims.

Examples

Example 1 – Synthesis of nanoparticles

Synthesis of gold nanoparticles having a corona comprising, e.g., carbohydrate ligands has been described previously (WO 2011/154711; and Lund *et al.*, 2011, Biomaterials Vol. 32 pp. 9776-9784, the entire contents of which are expressly incorporated herein by reference).

Briefly, preparation of amine alpha-gal gold nanoparticles involved the following procedure: To a mix of amine-mercapto hexaethylenglycol linker (structure **6** in WO 2011/154711) and alpha-galactose ligand (structure 3 in WO 2011/154711) in a ratio 1:1 (0.58 mmol, 3 eq.) in MeOH (49 mL) was added an aqueous solution of gold salt (7.86 mL, 0.19 mmol, 0.025M). The reaction was stirred during 30 seconds and then, an aqueous solution of NaBH4 (1N) was added in several portions (4.32 mL, 4.32 mmol). The reaction was shaken for 100 minutes at 900 rpm. After this time, the suspension was centrifuged 1 minute at 14000 rpm. The supernatant is removed and the precipitated was dissolved in 2 mL of water. Then, 2 mL of the suspension were introduced in two filters (AMICON, 10 KDa, 4 mL) and were centrifuged 5 minutes at 4500g. The residue in the filter was washed twice more with water. The final residue was dissolved in 80 mL of water.

A schematic representation of the resulting nanoparticles having a plurality of ligands in the ratio 1:1 of alpha-Gal:EG6NH2 *"NP-alpha-Gal(1)EG6NH2(1)"* is shown in Figure 11 of WO 2011/154711.

For the preparation of gold NPs manufacture was under laminar flow cabinet. All glass and plastic material (such as eppendorfs, vials and bottles) and solvent (water, HAc) were first sterilized in an autoclave. All other disposables (such as tips and filters) came pre-sterilized.

Binding of insulin and/or insulin analogue peptides to the nanoparticle corona was achieved essentially as described in Example 3 of WO 2011/154711.

Example 2 – Glucose and counter-hormone response to free and to nanoparticle-bound insulin and insulin analogues in vivo

In order to explore further the effects of nanoparticle-bound insulin and analogues thereof on glycaemic regulation *in vivo* and to compare and contrast with subcutaneously delivered, rapidly-acting insulin analogues administered as free peptide (i.e. not bound to nanoparticles), test items were administered to minipigs and pharmacodynamics responses evaluated. Animals were fasted overnight and then placed under anaesthesia.

Minipig plasma glucose was monitored over time and responses to free insulin lispro and to gold nanoparticle-bound insulin measured. Glucose concentration is shown plotted against time for 1.2 U/kg gold nanoparticle-bound insulin ("GNP-I"; filled squares); 0.3 U/kg GNPI (filled triangles); and 1.2 U/kg free insulin lispro (filled circles). The results indicate that for equal subcutaneous (s.c.) dose of analogue insulin lispro and GNP-regular insulin the analogue insulin lispro results in hypoglycaemia relative to nanoparticle-bound insulin.

Blood glucose clearance rates were evaluated in minipigs following administration of various control or test items. Figure 2A shows

results from control animals receiving no insulin; Figure 2B shows results from test animals receiving intravenous (i.v.) nanoparticle-bound insulin; Figure 2C shows results from control animals receiving 2.5 units of subcutaneous (s.c.) NovoRapid® insulin aspart; Figure 2D shows results from test animals receiving transbuccal GNP-regular insulin formulated in PharmFilm™. Each of these curves can be transformed, and early (K1), and late (K2) rate constants for glucose clearance calculated. The results show hypoglycemia for s.c. NovoRapid® (see Figure 2C), but no hypoglycemia for nanoparticle-bound insulin (see Figures 2B and 2D).

Next, the effect of subcutaneous (s.c.) injection of insulin in an insulin stress test was evaluated. Figure 3A shows results from a s.c. injection of 2.5 U/animal regular (Diosynth) free insulin upon administration to anaesthetized minipigs (1-squares; 2-circles; 3-triangles; and 4-inverted triangles) and blood glucose concentration plotted against time. Figure 3B shows results from a s.c. injection of 2.5 U/animal gold nanoparticle (GNP)-bound regular (Diosynth) insulin following administration to anaesthetized minipigs (1-squares; 2-circles; 3-triangles; and 4-inverted triangles) and blood glucose concentration plotted against time. Figure 3C shows results from a s.c. injection of 2.5 U/animal gold nanoparticle (GNP)-bound regular (Diosynth) insulin administered to anaesthetized minipigs (1-squares; 2-circles; 3-triangles; and 4-inverted triangles) with blood glucose concentration plotted against time. The results show that in these insulin stress tests neither s.c. administration of regular insulin or GNP-I insulin results in hypoglycaemia.

In a further experiment, the pharmacodynamics response to transbuccal nanoparticle-bound Apidra® was evaluated in anaesthetised minipigs and compared with the response to free Apidra®. Figure 4 shows an insulin stress test following s.c. injection of 0.15 IU/kg free Apidra® (analogue insulin glulisine; circles; n=8) or transbuccal delivery of gold nanoparticle (GNP)-bound Apidra® (analogue insulin glulisine; squares; n=8) in anesthetized minipigs. Blood glucose concentration is plotted against time. Significant features of the glucose response patterns

are indicated (e.g. the lack of neural recognition of hypoglycaemia and lack of counter-hormone responses are shown on the free Apridra® trace). These results show that s.c. administration of the rapidly-acting insulin analogue Apidra® results in hypoglycaemia, whereas transbuccal administration of GNP-bound Apidra® does not exhibit hypoglycaemia.

In a further experiment the possibility of induction of counter-hormone responses by s.c. administration of free Apidra® was investigated. Figures 5A-C show that s.c. free Apridra® does not induce counter-hormones during anaesthesia. Figure 5A: adrenalin (left-hand y-axis) and glucose (right-hand y-axis) concentrations are plotted against time for 0.15 U/kg s.c. administered Apidra® in anaesthetised minipigs; Figure 5B: glucagon (left-hand y-axis) and glucose (right-hand y-axis) concentrations are plotted against time for 0.15 U/kg s.c. administered Apidra® in anaesthetised minipigs; and Figure 5C growth hormone (left-hand y-axis) and glucose (right-hand y-axis) concentrations are plotted against time for 0.15 U/kg s.c. administered Apidra® in anaesthetised minipigs. The results show that, despite induction of hypoglycaemia, the Apidra® does not generate a counter-hormone response. S.c. administered Apidra® only induces counter-hormone release post-anesthesia; there is no induction of early hypoglycaemia recognition.

In a further experiment the possibility of induction of counter-hormone responses by transbuccally administered gold nanoparticle-bound Apridra® was investigated. Figures 6A and 6B show that transbuccally administered gold nanoparticle-bound Apridra® triggers a neural counter-hormone response in an insulin stress test in minipigs. Figure 6A: adrenalin (left-hand y-axis) and glucose (right-hand y-axis) concentrations are plotted against time for transbuccally administered, gold nanoparticle-bound Apidra® (TB-GNP-Apidra®); Figure 6B: growth hormone (GH) (left-hand y-axis) and glucose (right-hand y-axis) concentrations are plotted against time for transbuccally administered, gold nanoparticle-bound Apidra® (TB-GNP-Apidra®). The observed immediate rise of plasma adrenalin and

GH to TB-GNP-Apidra® could explain the quick rise in blood glucose post-TB-GNP-Apidra® administration.

All references cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each individual publication or patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

The specific embodiments described herein are offered by way of example, not by way of limitation. Any sub-titles herein are included for convenience only, and are not to be construed as limiting the disclosure in any way.

Claims:

1. A pharmaceutical composition comprising:

(i) free insulin or a free peptide analogue thereof; and

(ii) a plurality of nanoparticles, each of said nanoparticles comprising:

(a) a core comprising a metal and/or a semiconductor;

(b) a corona comprising a plurality of ligands covalently linked to the core, wherein at least one of said ligands comprises a carbohydrate moiety; and

(c) at least one insulin molecule or peptide analogue thereof non-covalently bound to the corona,

wherein the molar ratio of free insulin or free peptide analogue thereof to the nanoparticle-bound insulin or nanoparticle-bound peptide analogue thereof is in the range 1:1 to 100:1.

2. The pharmaceutical composition according to claim 1, wherein the peptide analogue of insulin has at least 70%, 80%, 90%, 95% or at least 99% amino acid sequence identity to the human insulin sequence of SEQ ID NO: 1 and is of between 40 and 60 amino acids in length.

3. The pharmaceutical composition according to claim 1 or claim 2, wherein the peptide analogue of insulin is selected from the group consisting of: insulin glulisine; insulin aspart; insulin lispro; NPH insulin; insulin glargine; insulin detemir; and insulin degludec.

4. The pharmaceutical composition according to any one of the preceding claims, wherein the nanoparticle-bound insulin or nanoparticle-bound insulin analogue induces an increase in plasma adrenalin concentration and/or plasma growth hormone concentration upon administration to a mammalian subject.

5. A method of regulating blood glucose concentration in a diabetic or pre-diabetic mammalian subject, said method comprising administering to the subject an effective amount of the pharmaceutical composition according to any one of claims 1 to 4.

6. A method of reducing the incidence of hypoglycemic adverse events in an insulin-dependent diabetic or pre-diabetic mammalian subject, said method comprising:

administering, simultaneously, sequentially or concurrently with a dose of insulin or a peptide analogue thereof, an effective amount of the pharmaceutical composition comprising a plurality of nanoparticles, each of said nanoparticles comprising:

(a) a core comprising a metal and/or a semiconductor;

(b) a corona comprising a plurality of ligands covalently linked to the core, wherein at least one of said ligands comprises a carbohydrate moiety; and

(c) at least one insulin molecule or peptide analogue thereof non-covalently bound to the corona.

7. The method according to claim 6, wherein administration is sequential, and wherein the dose of free insulin or peptide analogue thereof is administered prior to or after the dose of nanoparticle-bound insulin or analogue thereof.

8. The method according to claim 7, wherein of free insulin or peptide analogue thereof and the dose of nanoparticle-bound insulin or analogue thereof are administered between 1 second and 1 hour apart, between 1 minute and 10 minutes apart, or between 2 minutes and 5 minutes apart.

9. A pharmaceutical composition as defined in any one of claims 1 to 1 to 4 for use in medicine.

10. A pharmaceutical composition as defined in any one of claims 1 to 1 to 4 for use in a method as defined in any one of claims 5 to 8.

11. The pharmaceutical composition or method as defined in any one of the preceding claims, wherein the corona comprises one or more carbohydrate ligands covalently attached to the core via a linker.

12. The pharmaceutical composition or method as defined in any one of the preceding claims, wherein the insulin or analogue thereof comprises or consists of an amino acid A chain having at least 70%, 80%, 90%, 95% or 99% amino acid sequence identity with the full-length amino acid sequence set forth as SEQ ID NO: 1, and an amino acid B chain having at least 70%, 80%, 90%, 95% or 99% amino acid sequence identity with any one of the full-length amino acid sequences set forth as SEQ ID NOS: 2-5, wherein the A and B chains are linked.

13. The pharmaceutical composition or method according to claim 12, wherein the analogue of insulin comprises a B chain that has up to 1, 2, 3, 4 or 5 amino acid changes by substitution, addition and/or deletion as compared with any one of the full-length B chain amino acid sequences set forth in SEQ ID NOs: 2-5.

14. The pharmaceutical composition or method according to any one of the preceding claims, wherein the analogue of insulin comprises a fast-acting insulin analogue.

15. The pharmaceutical composition or method according to any one of the preceding claims, wherein the insulin analogue is selected from the group consisting of: insulin lispro, insulin aspart, and insulin glulisine.

16. The pharmaceutical composition or method according to any one of the preceding claims, wherein the free peptide insulin analogue and the nanoparticle-bound insulin analogue are the same insulin analogue or are different insulin analogues.

17. The pharmaceutical composition or method according to any one of the preceding claims, wherein the corona comprises at least 5, 10, 20 or at least 50 ligands per core.

18. The pharmaceutical composition or method according to any one of the preceding claims, wherein the average number of nanoparticle-bound insulin peptides or nanoparticle-bound insulin

analogue peptides per nanoparticle core is at least 1, 2, 3, 4, 5, 10, 15, 20, 30 or at least 50 or more.

19. The pharmaceutical composition or method according to claim 18, wherein there are, on average, between 10 and 30 glulisine peptide molecules bound per nanoparticle core.

20. The pharmaceutical composition or method according to any one of the preceding claims, wherein said at least one ligand comprising a carbohydrate moiety is selected from the group consisting of: 2´-thioethyl-α-D-galactopyranoside, 2´-thioethyl-β-D-glucopyranoside, 2´-thioethyl-2-acetamido-2-deoxy-β-D-glucopyranoside, 5´-thiopentanyl-2-deoxy-2-imidazolacetamido-α,β-D-glucopyranoside and 2´-thioethyl-α-D-glucopyranoside, wherein said at least one ligand comprising a carbohydrate moiety is covalently linked to the core via its sulphur atom.

21. The pharmaceutical composition or method according to claim 20, wherein said plurality of ligands covalently linked to the core may comprise at least a first ligand and a second ligand, wherein the first and second ligands are different.

22. The pharmaceutical composition or method according to claim 21, wherein:

(a) said first ligand comprises 2´-thioethyl-α-D-galactopyranoside and said second ligand comprises 1-amino-17-mercapto-3,6,9,12,15,-pentaoxa-heptadecanol;

(b) said first ligand comprises 2´-thioethyl-β-D-glucopyranoside or 2´-thioethyl-α-D-glucopyranoside and said second ligand comprises 5´-thiopentanyl-2-deoxy-2-imidazolacetamido-α,β-D-glucopyranoside;

(c) said first ligand comprises 2´-thioethyl-β-D-glucopyranoside or 2´-thioethyl-α-D-glucopyranoside and said second

ligand comprises 1-amino-17-mercapto-3,6,9,12,15,-pentaoxa-heptadecanol; or

(d) said first ligand comprises 2´-thioethyl-2-acetamido-2-deoxy-β-D-glucopyranoside and said second ligand comprises 1-amino-17-mercapto-3,6,9,12,15,-pentaoxa-heptadecanol,

and wherein said first and second ligands are covalently linked to the core via their respective sulphur atoms.

23. The pharmaceutical composition or method according to claim 21 or claim 22, wherein the first ligand and said second ligand are present on the nanoparticle in a molar ratio in the range of 1:40 to 40:1, 1:10 to 10:1, or 1:2 to 2:1.

24. The pharmaceutical composition or method according to any one of the preceding claims, wherein the nanoparticle comprises a component having a divalent state.

25. The pharmaceutical composition or method according to claim 24, wherein said component having a divalent component is selected from: Zn^{2+} and ZnO.

26. The pharmaceutical composition or method according to any one of the preceding claims, wherein the diameter of the core of the nanoparticle is in the range 1 nm to 5 nm.

27. The pharmaceutical composition or method according to any one of the preceding claims, wherein the diameter of the nanoparticle including its ligands is in the range 2 nm to 50 nm, 3 nm to 30 nm, 4 nm to 20 nm, or 5 nm to 15 nm.

28. The pharmaceutical composition or method according to any one of the preceding claims, wherein the core comprises a metal selected from the group consisting of: Au, Ag, Cu, Pt, Pd, Fe, Co, Gd and Zn, or any combination thereof.

29. The pharmaceutical composition or method according to any one of the preceding claims, wherein the core comprises a semiconductor.

30. The pharmaceutical composition or method according to any one of the preceding claims, wherein said plurality of nanoparticles comprises 100, 1000, 100000, or more, of said nanoparticles, wherein at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% of the nanoparticles in said composition have at least one insulin or insulin analogue peptide bound.

31. The pharmaceutical composition or method according to any one of the preceding claims, wherein said plurality of nanoparticles are formulated in a carrier, a solution, a polymer, a powder, or a cream, in which the nanoparticles and bound insulin and/or insulin analogue peptides are suspended and/or embedded.

32. The pharmaceutical composition or method according to claim 31, wherein the nanoparticle formulation is in the form of a patch or film for delivery to or across skin, mouth, cheek, vagina, rectum or in the form of a spray for delivery into the mouth, nose, lungs or the rectum or vagina.

33. The pharmaceutical composition or method according to any one of the preceding claims, wherein the nanoparticles having insulin or analogue thereof bound and the free peptide insulin or free peptide analogue thereof are in a single package, container or carrier.

34. The pharmaceutical composition or method according to any one of the preceding claims, wherein the composition is for administration to or the method comprises administration to a human subject having type 1 diabetes, type 2 diabetes or prediabetes.

35. The pharmaceutical composition or method according to any one of the preceding claims, wherein the composition is for administration to or the method comprises administration to a subject having hypoglycaemia.

36. The pharmaceutical composition or method according to claim 35, wherein said hypoglycaemia is a temporary state resulting from excess administration of insulin or insulin analogue, or from insufficient food intake following administration of insulin or insulin analogue, or from a situation in which administration of insulin or insulin analogue and intake of food have been poorly timed.

37. The pharmaceutical composition or method according to any one of the preceding claims, wherein the composition is for self-administered or the method comprises self-administration.

38. An article of manufacture comprising:

a pharmaceutical composition as defined in any one of the preceding claims;

a container for housing the pharmaceutical composition; and

an insert and/or label.

39. The article of manufacture according to claim 38, wherein the insert and/or label provide instructions, dosage and/or administration information relating to the use of the pharmaceutical composition in a method of managing blood glucose concentration, glycaemic control and/or a method of managing or treating diabetes.

40. A process for producing a pharmaceutical composition as defined in any one of the preceding claims, comprising:

providing a nanoparticle comprising a core comprising a metal and/or a semiconductor and a corona comprising a plurality of ligands covalently linked to the core, wherein said ligands comprise one or more carbohydrate-containing ligands;

contacting the nanoparticle with at least one insulin peptide and/or insulin analogue peptide under conditions which allow the at least one insulin peptide and/or insulin analogue peptide to bind to the corona of the nanoparticle; and

combining the resulting nanoparticle having insulin peptide and/or insulin analogue peptide bound thereto with insulin peptide and/or insulin analogue peptide that is not bound to a nanoparticle.

41. The process according to claim 40, wherein said combining step comprises including the nanoparticle having insulin peptide and/or insulin analogue peptide bound thereto in a housing or container with the insulin peptide and/or insulin analogue peptide that is not bound to a nanoparticle.

42. The process according to claim 40 or claim 41, wherein the insulin analogue peptide is as defined in any one of the preceding claims.

43. The process according to any one of claims 40 to 42, comprising an earlier step of producing the nanoparticle, said earlier step comprising: combining a solution comprising one or more derivatised carbohydrate moieties with a solution comprising a core-forming material and with a reducing agent, thereby causing the nanoparticle to self-assemble.

44. The process according to claim 43, wherein said derivatised carbohydrate moieties comprise thioethyl-alpha galactose, said solution comprising a core-forming material comprise gold III chloride and said reducing agent comprises sodium borohydride.

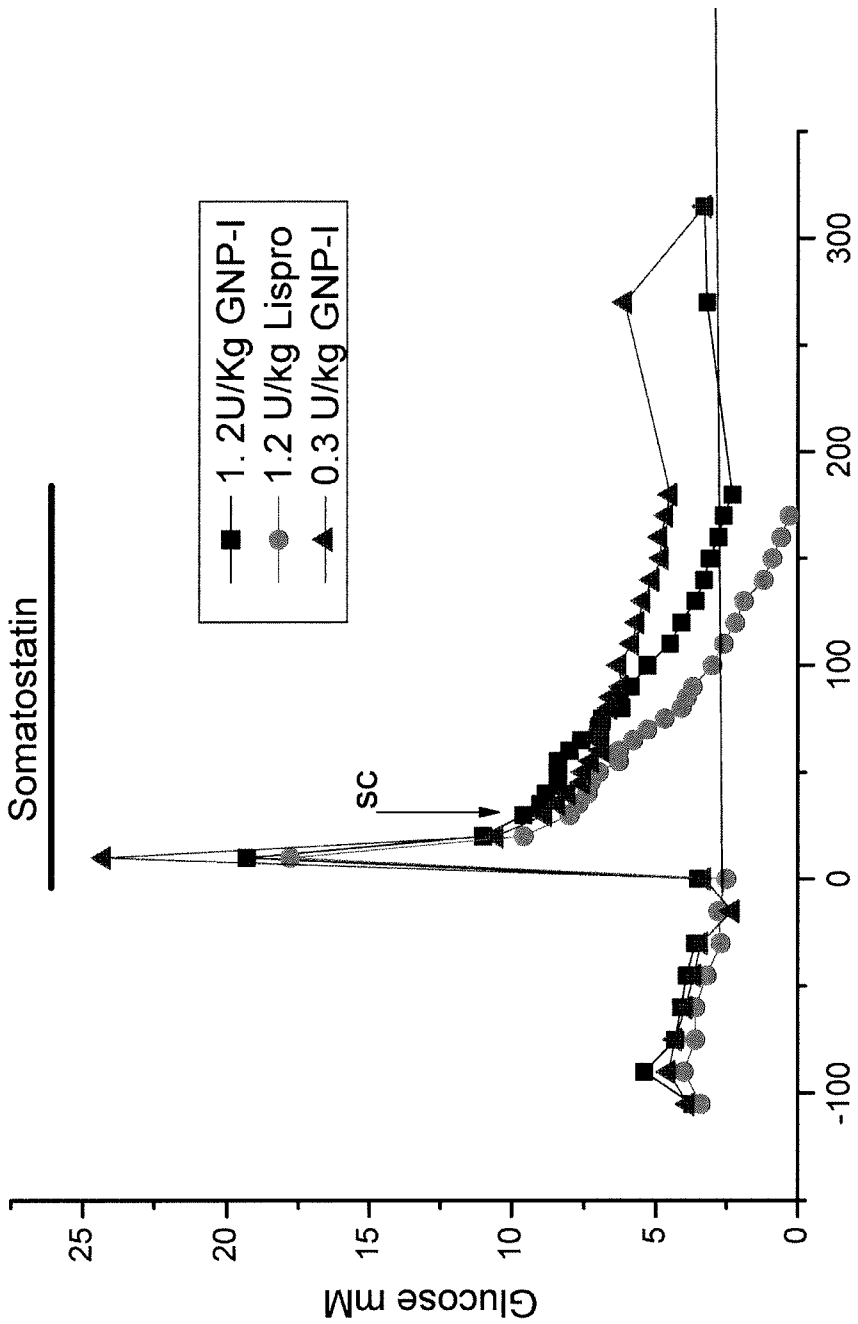
Somatostatin
sc
1. 2U/Kg GNP-I
1.2 U/kg Lispro
0.3 U/kg GNP-I
Glucose mM
25
20
15
10
5
0
-100
0
100
200
300
Time (min)

Figure 1

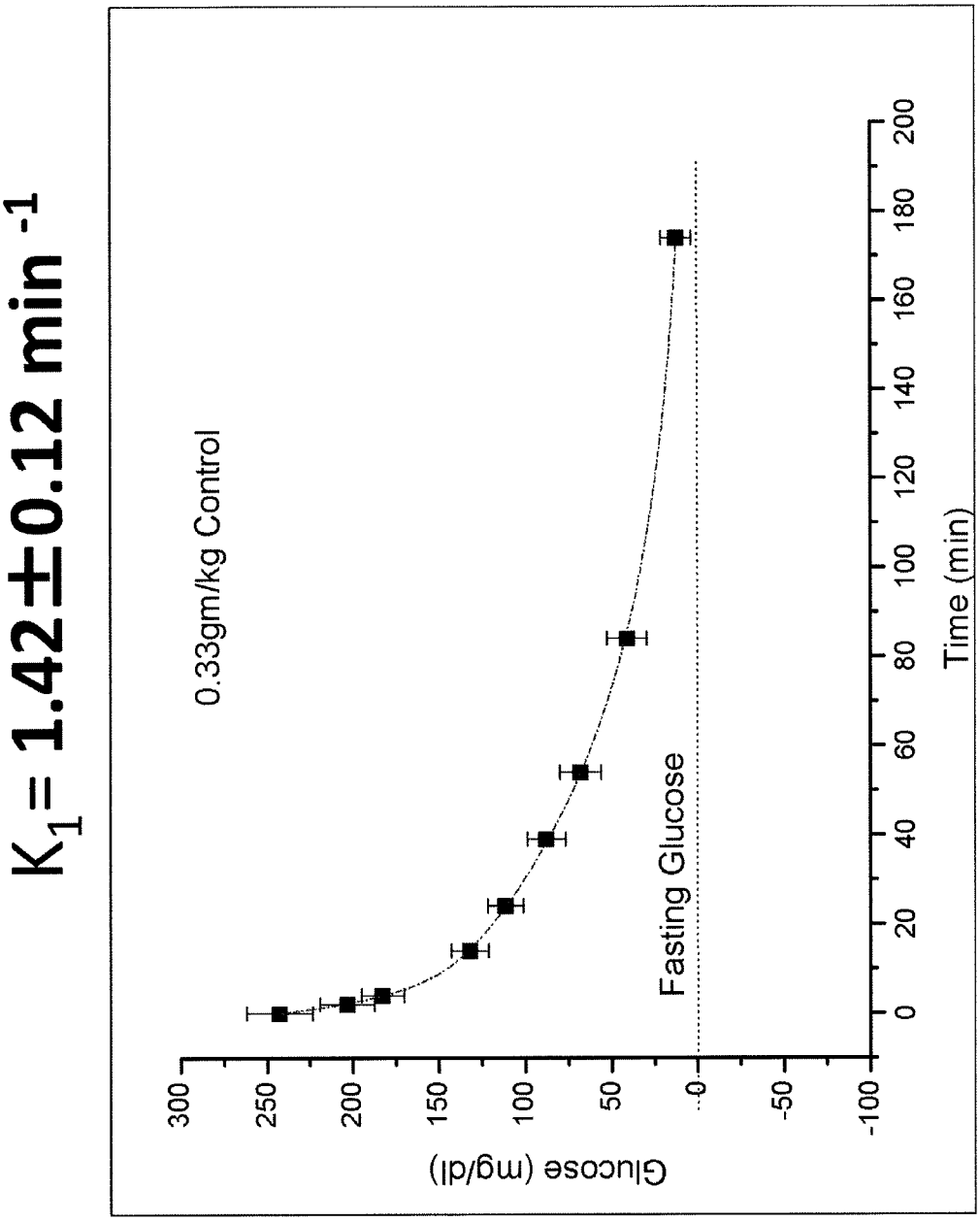
$K_1 = 1.42 \pm 0.12\ min^{-1}$
0.33gm/kg Control
Fasting Glucose
Glucose (mg/dl)
Time (min)
300
250
200
150
100
50
0
-50
-100
0
20
40
60
80
100
120
140
160
180
200

Figure 2A

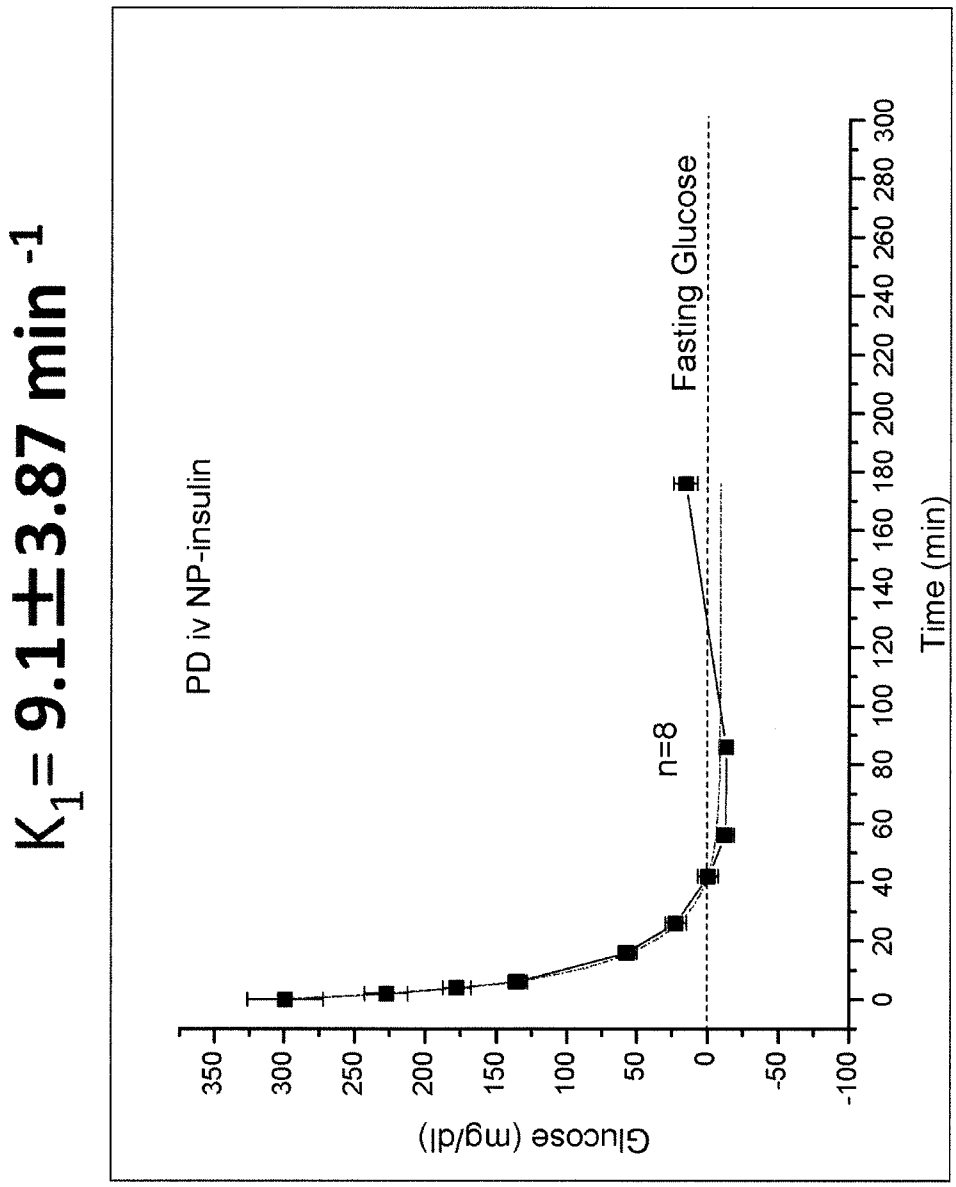
$K_1 = 9.1 \pm 3.87\ min^{-1}$
PD iv NP-insulin
n=8
Fasting Glucose
Glucose (mg/dl)
Time (min)
350
300
250
200
150
100
50
0
-50
-100
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300

Figure 2B

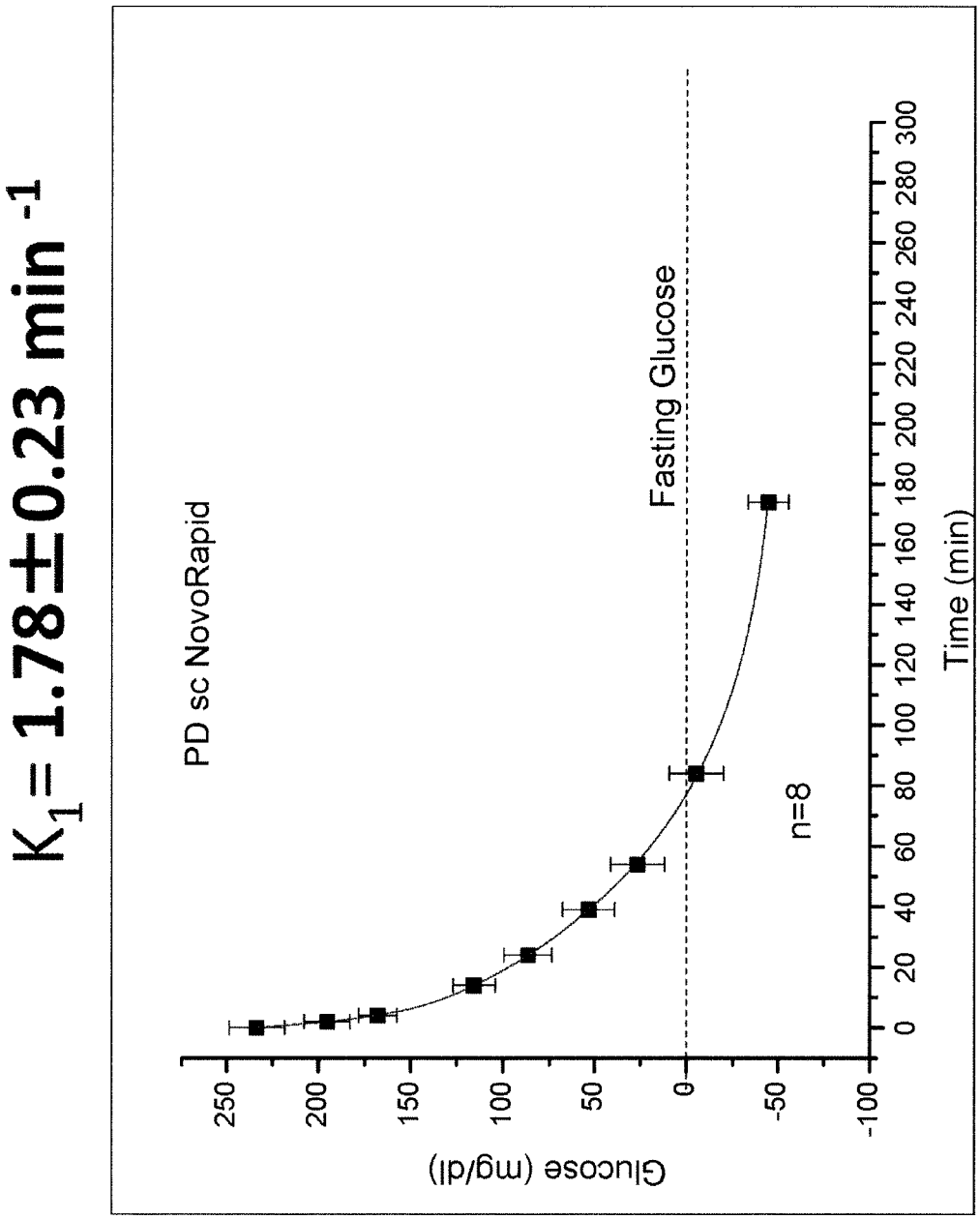
$K_1 = 1.78 \pm 0.23\ min^{-1}$
PD sc NovoRapid
Fasting Glucose
n=8
Glucose (mg/dl)
Time (min)
250
200
150
100
50
0
-50
-100
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300

Figure 2C

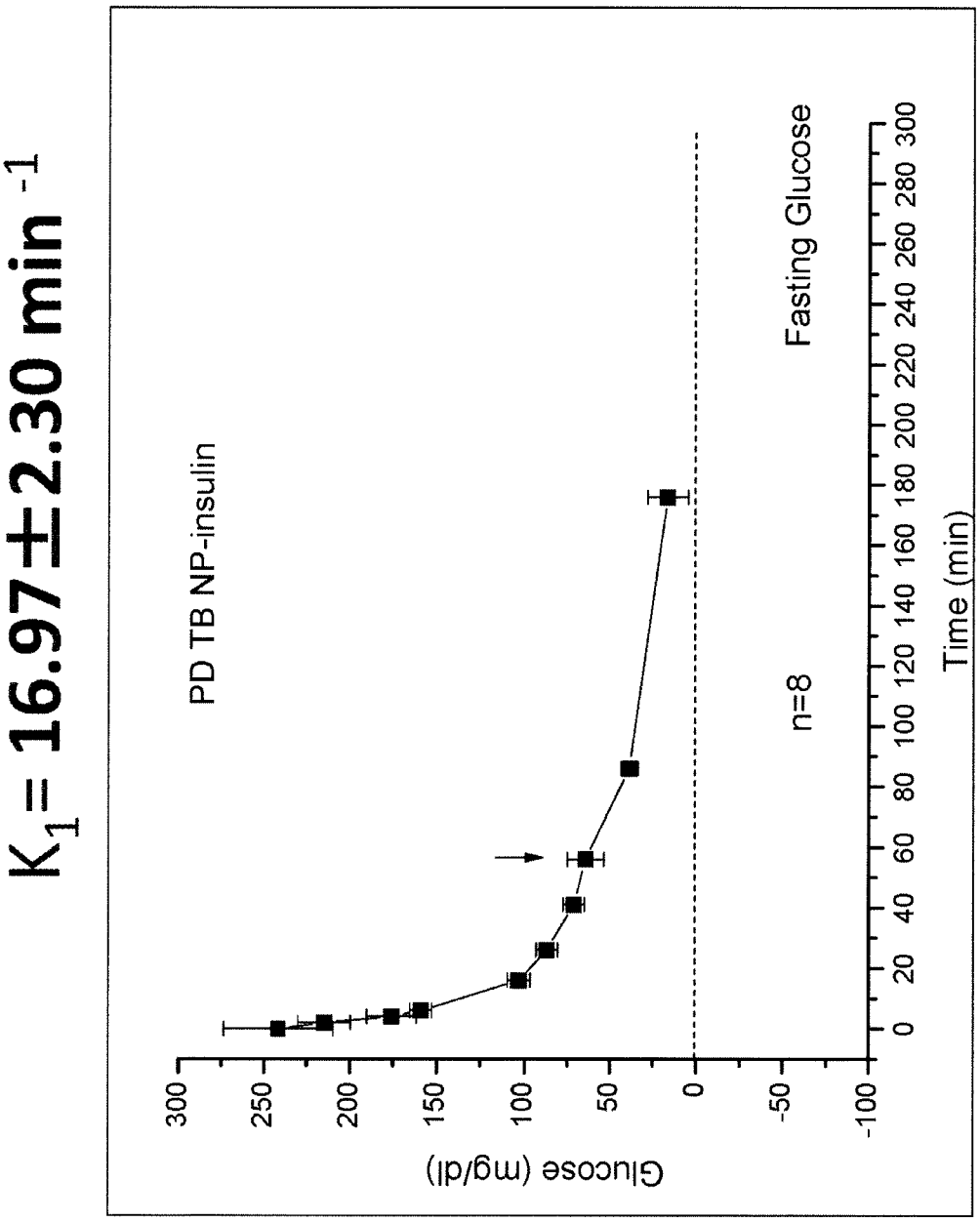
$K_1 = 16.97 \pm 2.30\ min^{-1}$
PD TB NP-insulin
Fasting Glucose
n=8
Glucose (mg/dl)
Time (min)
300
250
200
150
100
50
0
-50
-100
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300

Figure 2D

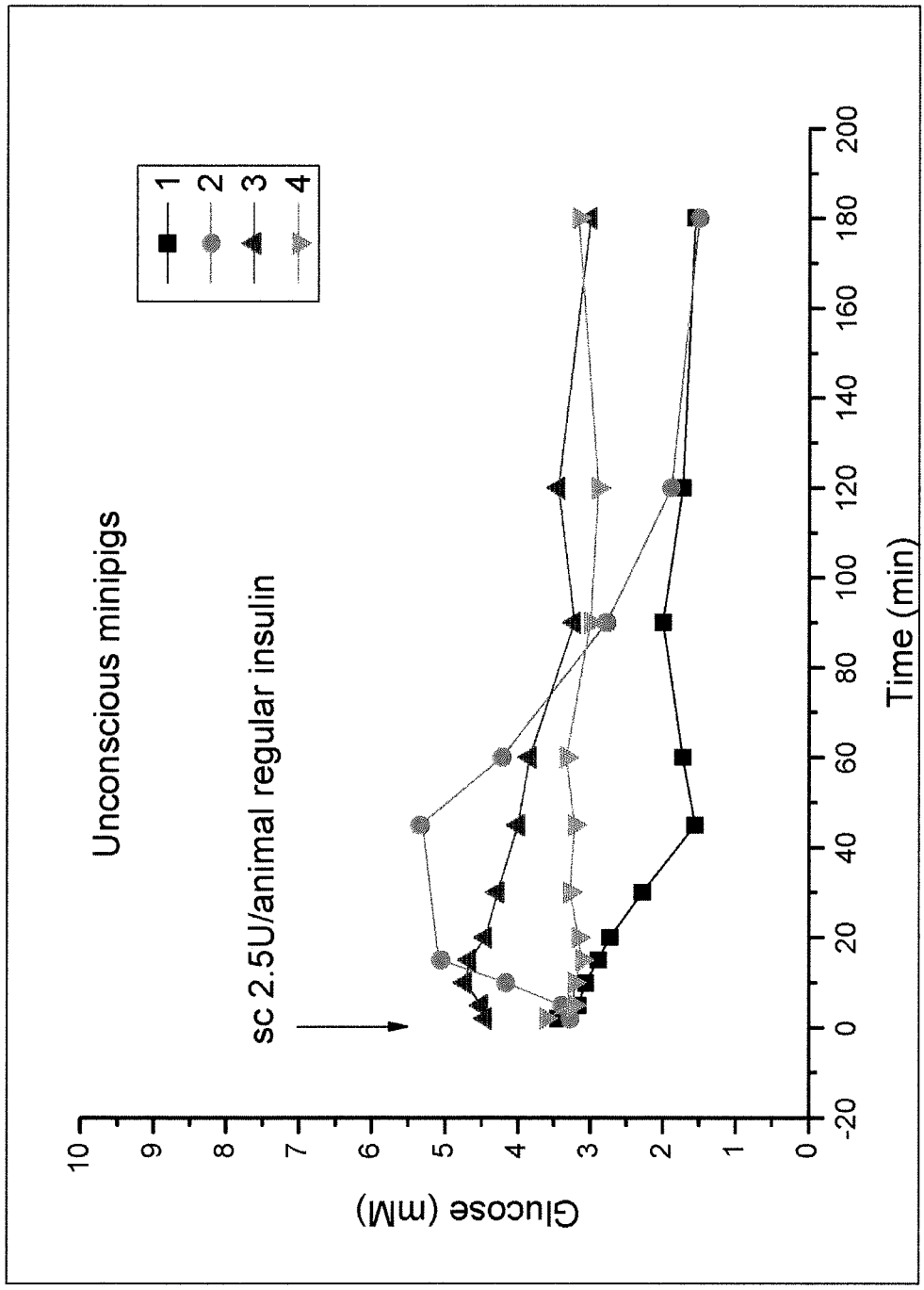
Unconscious minipigs
sc 2.5U/animal regular insulin
1
2
3
4
Glucose (mM)
Time (min)
10
9
8
7
6
5
4
3
2
1
0
-20
0
20
40
60
80
100
120
140
160
180
200

Figure 3A

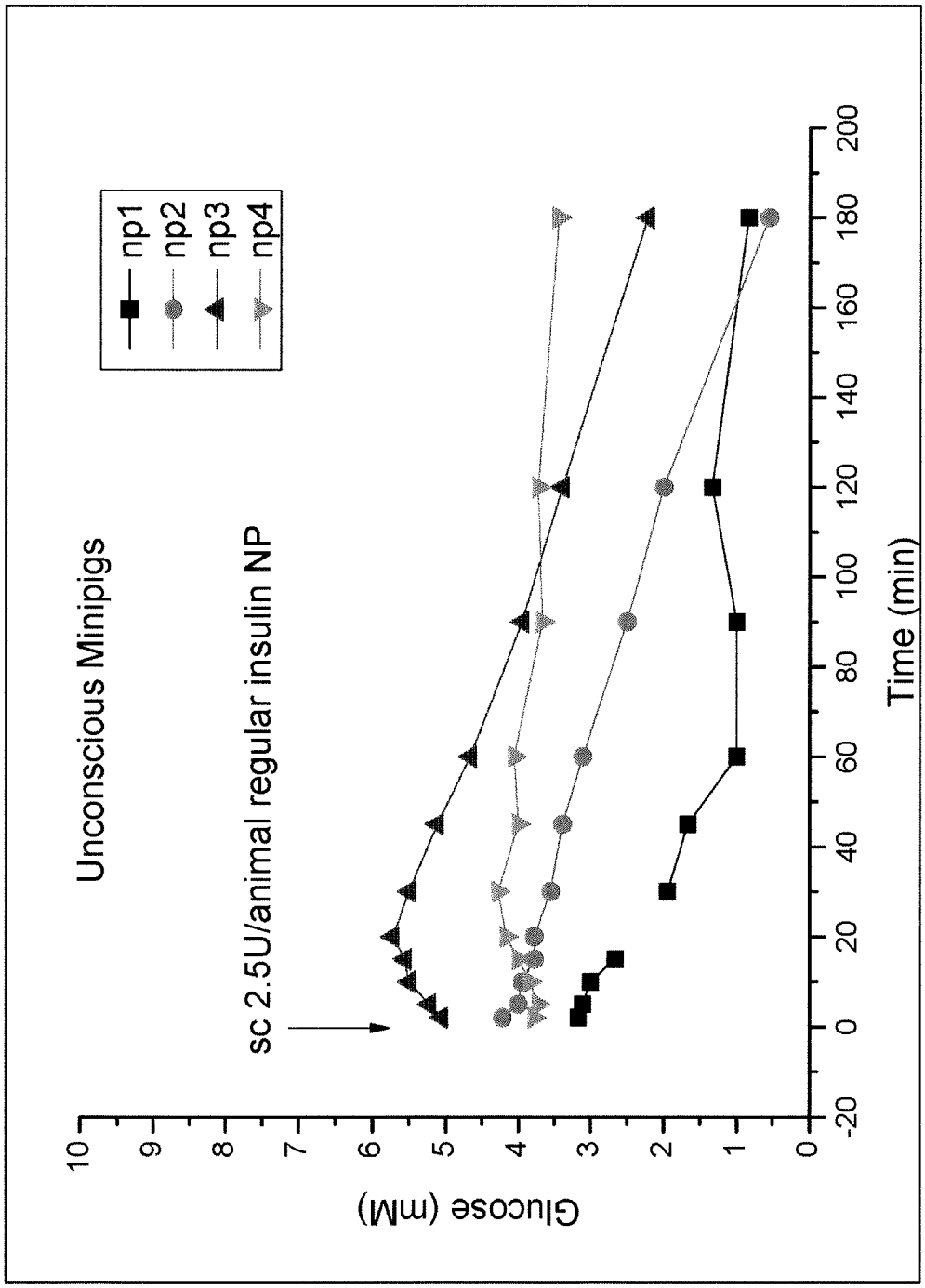
Unconscious Minipigs
sc 2.5U/animal regular insulin NP
np1
np2
np3
np4
Glucose (mM)
Time (min)
10
9
8
7
6
5
4
3
2
1
0
-20
0
20
40
60
80
100
120
140
160
180
200

Figure 3B

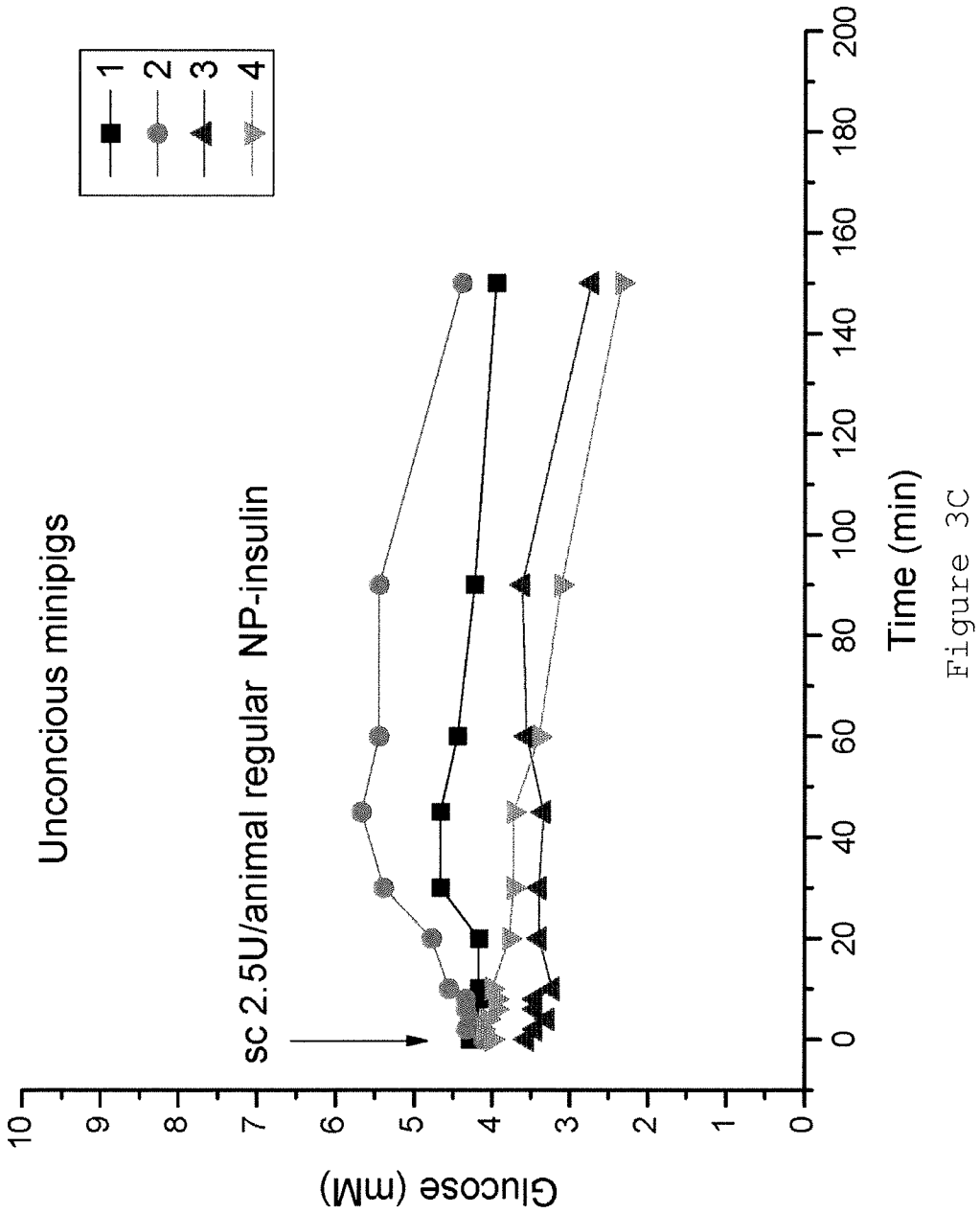
Unconcious minipigs
sc 2.5U/animal regular NP-insulin
1
2
3
4
Glucose (mM)
Time (min)
0
1
2
3
4
5
6
7
8
9
10
0
20
40
60
80
100
120
140
160
180
200

Figure 3C

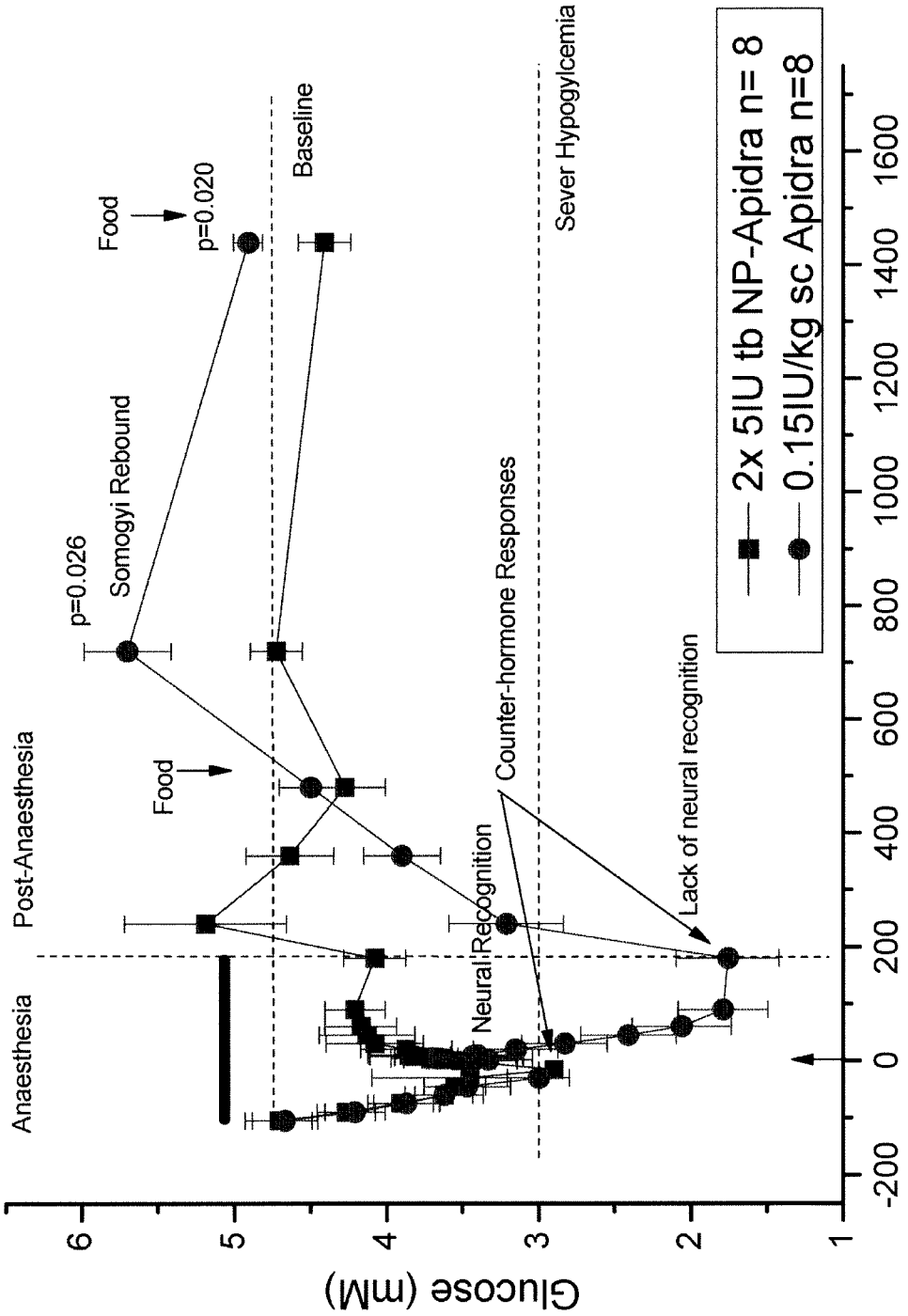
Glucose (mM)
Time (min)
2x 5IU tb NP-Apidra n= 8
0.15IU/kg sc Apidra n=8
Anaesthesia
Post-Anaesthesia
Food
p=0.026
Somogyi Rebound
Food
p=0.020
Baseline
Neural Recognition
Counter-hormone Responses
Lack of neural recognition
Sever Hypogylcemia

Figure 4

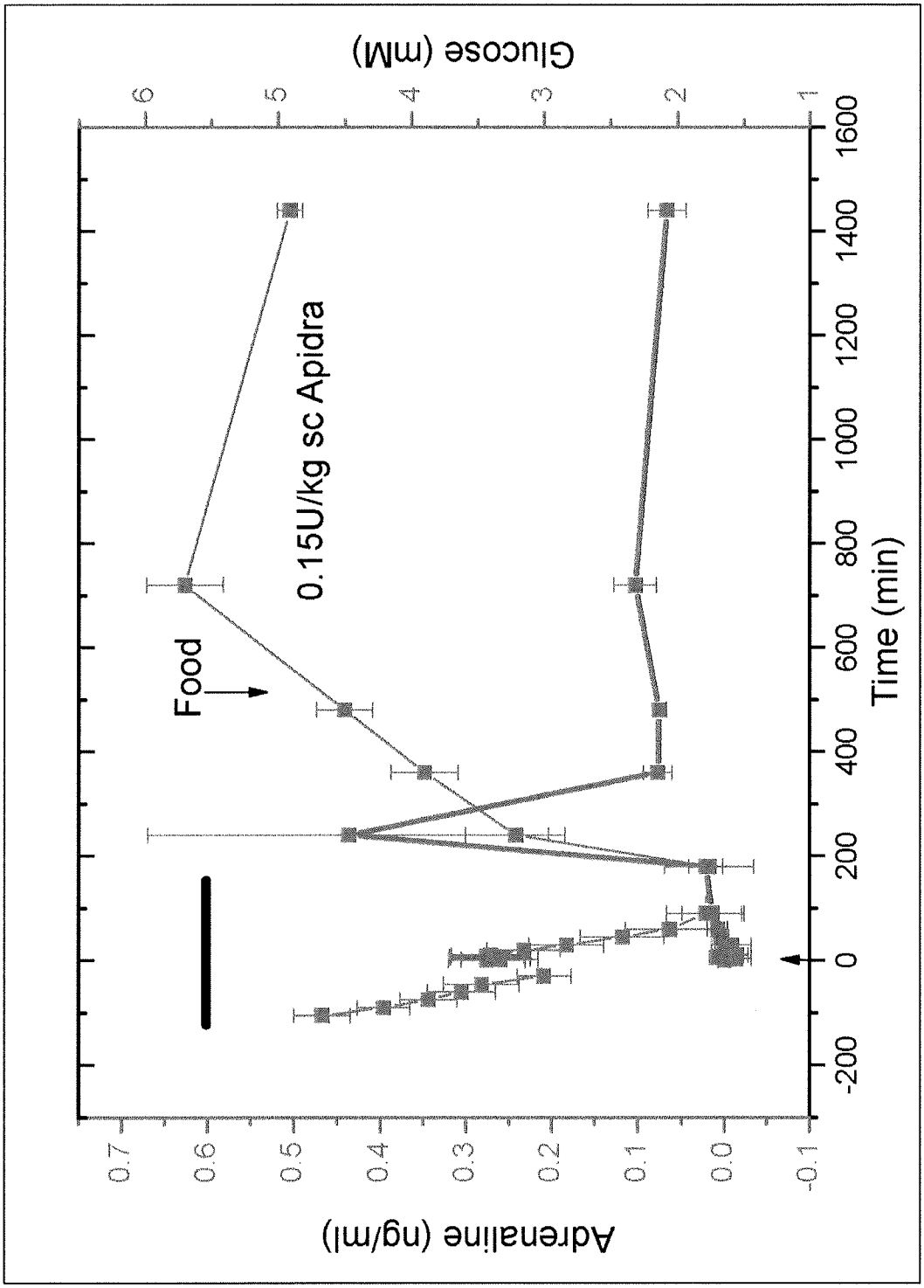

Figure 5A

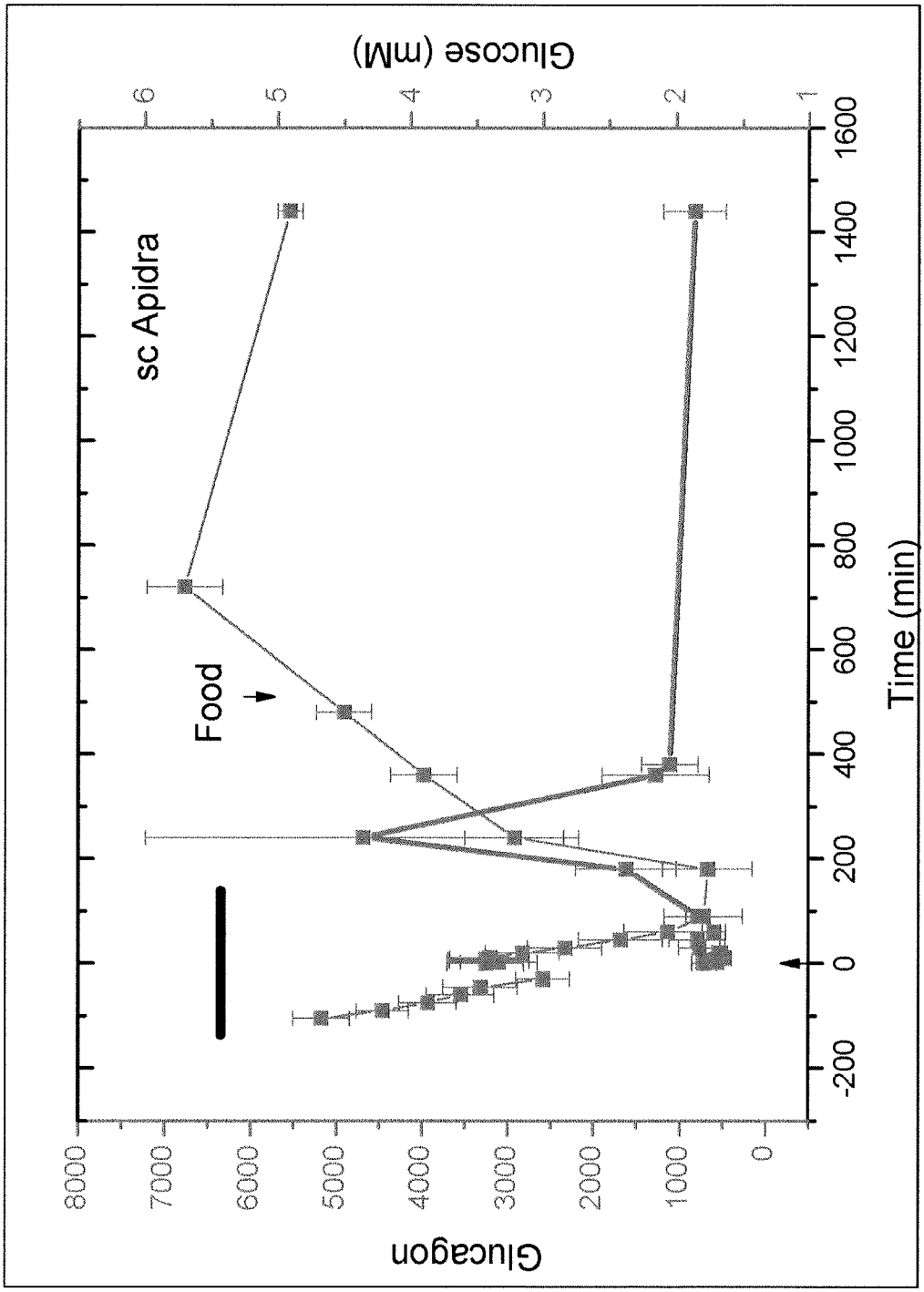
Glucagon
Glucose (mM)
Time (min)
sc Apidra
Food
8000
7000
6000
5000
4000
3000
2000
1000
0
6
5
4
3
2
1
-200
0
200
400
600
800
1000
1200
1400
1600

Figure 5B

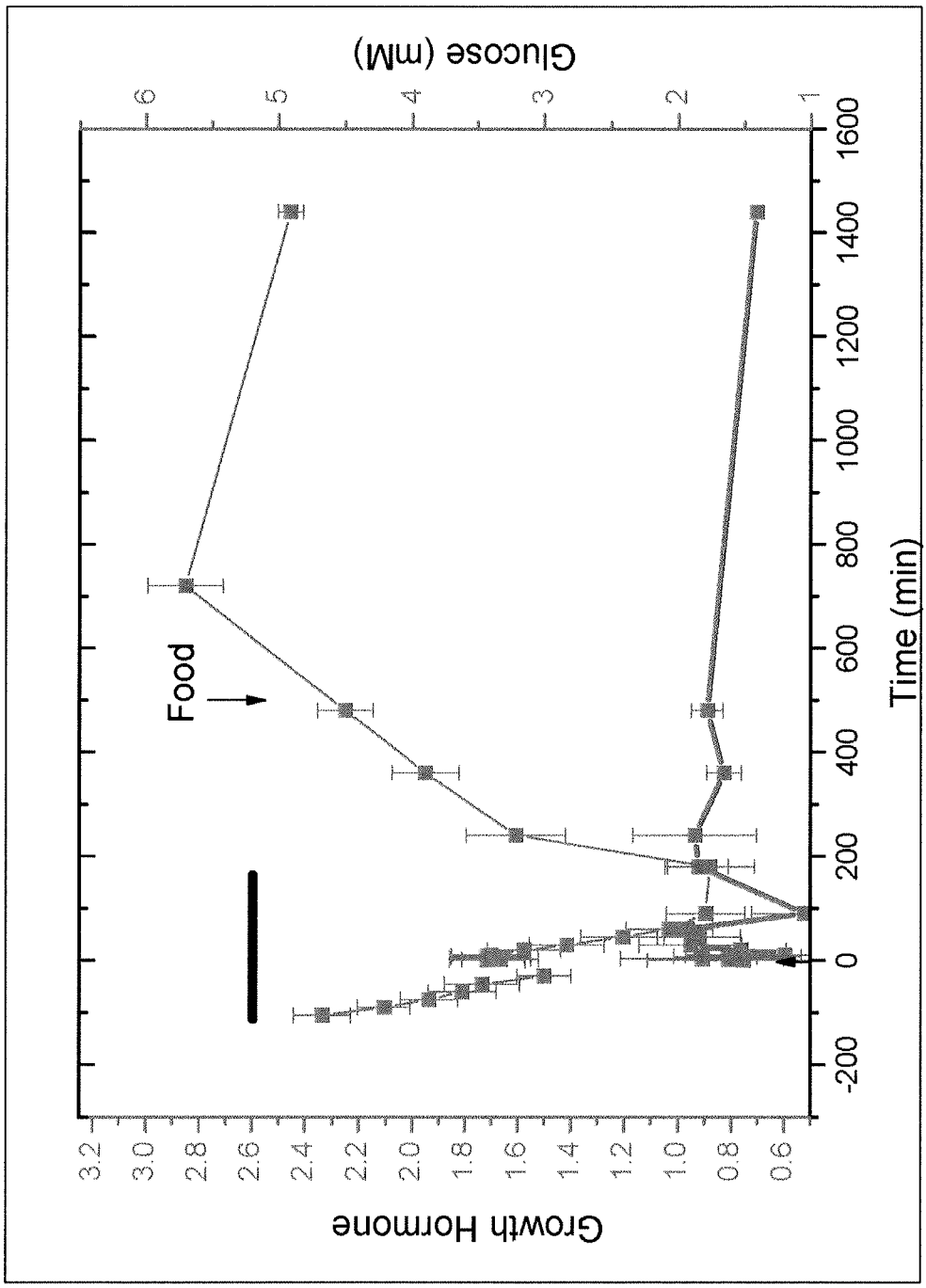
Growth Hormone
Glucose (mM)
Time (min)
Food
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
6
5
4
3
2
1
-200
0
200
400
600
800
1000
1200
1400
1600

Figure 5C

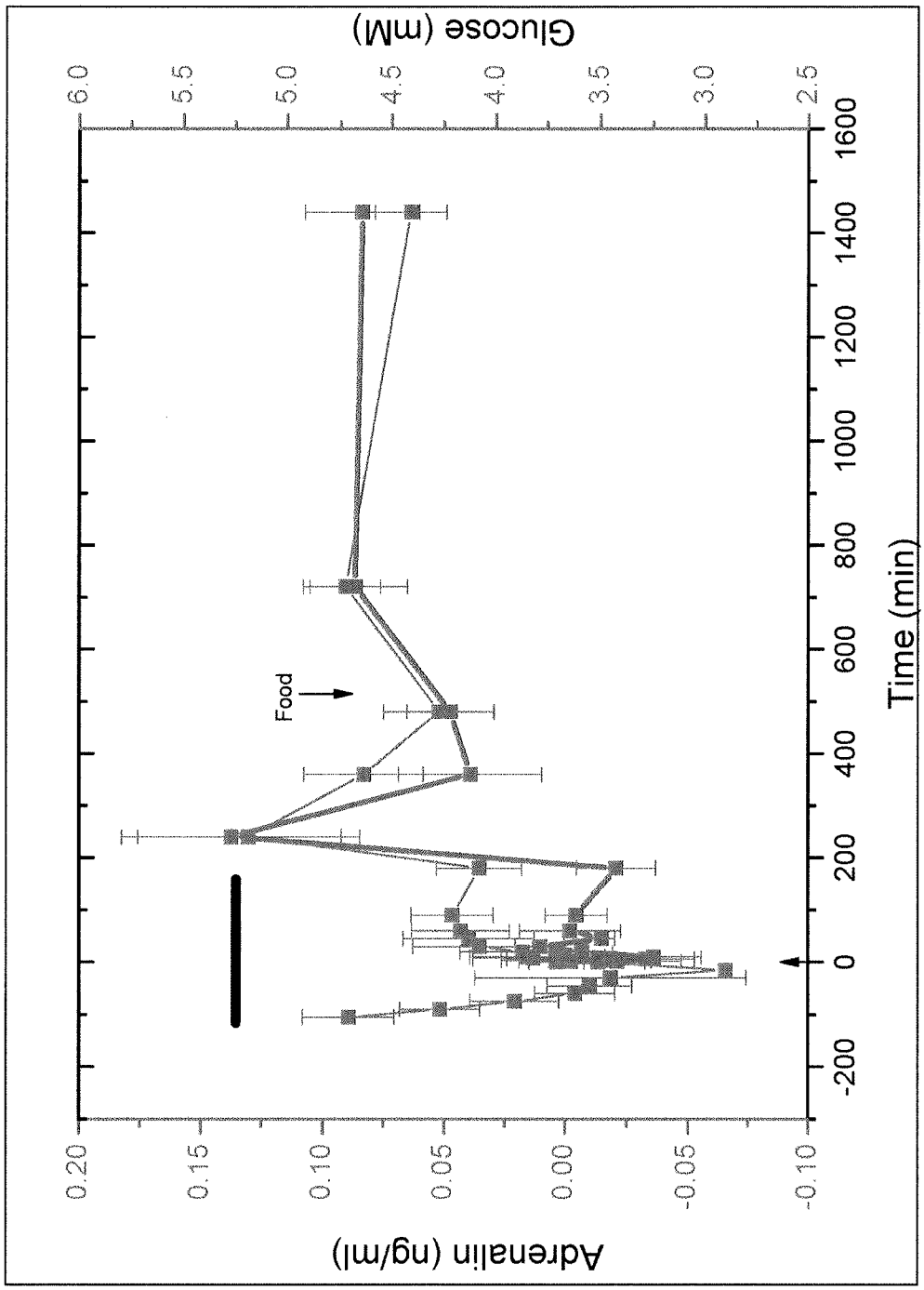
Glucose (mM)
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
Food
Time (min)
-200
0
200
400
600
800
1000
1200
1400
1600
Adrenalin (ng/ml)
0.20
0.15
0.10
0.05
0.00
-0.05
-0.10

Figure 6A

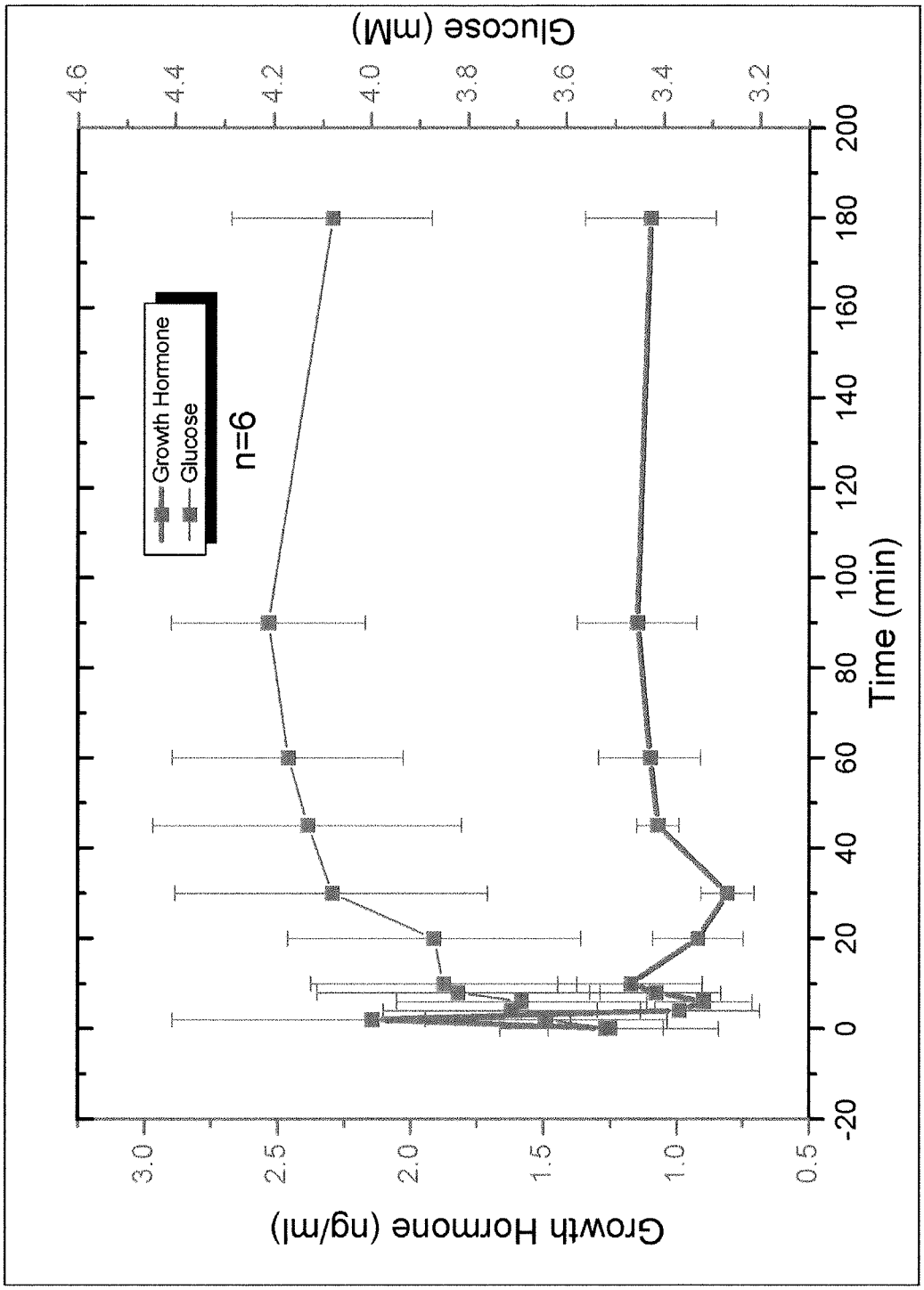
Growth Hormone (ng/ml)
Glucose (mM)
Time (min)
Growth Hormone
Glucose
n=6
3.0
2.5
2.0
1.5
1.0
0.5
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
-20
0
20
40
60
80
100
120
140
160
180
200

Figure 6B

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2015/050210

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/48 A61P3/10
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/154711 A1 (MIDATECH LTD [GB]; RADEMACHER THOMAS [GB]; WILLIAMS PHILLIP [GB]; BACH) 15 December 2011 (2011-12-15) abstract page 16, line 23 - page 18, line 3 claims; examples 3,5 figures 12,17 -----	1-44
X	WO 2011/156711 A1 (SCHOBEL ALEXANDER M [US]; MYERS GARRY L [US]; KENDALL KEITH JOSEPH [US) 15 December 2011 (2011-12-15) abstract page 3, line 8 - line 21 page 14, line 7 - line 20; claims; examples 3,5 figures 12,17 ----- -/--	1-44

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 April 2015	28/04/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Hoff, Philippe

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2015/050210

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/170828 A1 (MONOSOL RX LLC [US]; MIDATECH LTD [GB]; WILLIAMS PHILLIP [GB]; RADEMAC) 13 December 2012 (2012-12-13) abstract page 1, line 15 - line 18 page 16, line 25 - page 17, line 30; claims; examples 3,5,14-16 figures 12,17	1-44
X	A.G. Barrientos: "Transbuccal Insulin-Glyconanoparticles: An Alternative to Injectable Insulin", , July 2012 (2012-07), XP002738283, Retrieved from the Internet: URL:http://www.egsf.org/assets/meeting-report.pdf [retrieved on 2015-04-09] abstract	1-44
A	DEVIKA R BHUMKAR ET AL: "Chitosan Reduced Gold Nanoparticles as Novel Carriers for Transmucosal Delivery of Insulin", PHARMACEUTICAL RESEARCH, KLUWER ACADEMIC PUBLISHERS-PLENUM PUBLISHERS, NL, vol. 24, no. 8, 23 March 2007 (2007-03-23) , pages 1415-1426, XP019507241, ISSN: 1573-904X, DOI: 10.1007/S11095-007-9257-9 the whole document	1-44

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2015/050210

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		JP 2015038091 A	26-02-2015
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		JP 2014522412 A	04-09-2014
		WO 2012170828 A1	13-12-2012