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(54) Benævnelse: **EXENDIN-4-ANALOG PEGYLERET MED POLYETHYLENGLYCOL ELLER ET DERIVAT DERAF, FREMGANGSMÅDE TIL FREMSTILLING DERAF OG FARMACEUTISK SAMMENSÆTNING TIL FOREBYGGELSE ELLER BEHANDLING AF DIABETES, INDEHOLDENDE SAMME SOM AKTIV BESTANDDEL**

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DESCRIPTION

TECHNICAL FIELD

[0001] The present disclosure relates to an exendin-4 analogue PEGylated with polyethylene glycol or a derivative thereof, a preparation method thereof, and a pharmaceutical composition for prevention or treatment of diabetes containing the same as an active ingredient as set out in the appended claims.

BACKGROUND ART

[0002] Among pharmaceutical technologies, PEGylation of peptides and proteins for the purpose of treatment is the most effective technology. PEGylation of peptides and proteins increases molecular weight thereof, protein degradation site defense and immunogenicity site defense, which consequently increases half-life of in vivo medications and reduce immunogenicity of peptides and proteins. Therefore, PEGylation technology has an effect of increasing treatment effect by solving problems of original medications, and due to such strength, serves an important role in increasing effects of PEGylated peptide and protein medication delivery system.

[0003] Also, peptides and proteins increase treatment effect by covalently bonding with polyethylene glycol (PEG). Such technology increases molecular weight, defense of a metabolism site and inhibition of an immunogenicity site, increasing in vivo half-life and stability and reducing immunogenicity. Furthermore, kidney excretion of peptides and proteins bound with PEG is reduced due to the increase of molecular weights of peptides and proteins by PEG, so that PEGylation has advantages of increasing effects in both pharmacokinetically and pharmacodynamically.

[0004] PEGylation reacting sites of peptides and proteins are randomly dispersed and are occasionally close to bioactive sites. However, traditional PEGylation employs nonspecific PEGylation methods that do not consider PEG reacting site, number of PEG bonds and biological activity. However, such a nonspecific PEGylation method reduces treatment effects by bringing insufficient conformation by producing various branched type PEG-bonded isomers that have different physiochemical, biological and pharmacokinetic characteristics. Specific PEGylation methods have been studied to solve such problems, and recently the specific PEGylation methods are rapidly developing to become a method of maximizing medications' treatment effects as genetic engineering technology and selective functional group introducing technology are quickly developing. In a related art, a study of selectively binding PEG into N-terminal site after removing a reaction site by substituting primary amine site with different amino acid using genetic engineering method for granulocyte stimulating factor (G-CSF) and tumor necrosis factor receptor has been conducted previously.

[0005] Also, studies using a technology that selectively PEGylates substituent after having introduced a specific substituent using genetic engineering methods and substitution technology for medications such as staphylokinase, interferon a-2, antibody single chain fragment variable (ScFv), have been conducted.

[0006] WO 2004/022004 to Bayer Pharmaceuticals Corp describes modified GLP-1 receptor agonists comprising a GLP-1 receptor agonist linked to a polyethylene glycol polymer having a molecule weight of greater than 30 kD, and related formulations and dosages and methods of administration thereof for

therapeutic purposes. More particularly, these modified GLP-1 receptor agonists, compositions and methods are taught to be useful in providing a treatment option for those individuals afflicted with a metabolic disorder such as diabetes and prediabetic states such as impaired glucose tolerance, and impaired fasting glucose, by inducing glucose-dependent insulin secretion, without reducing gastrointestinal motility.

[0007] Exendin-4 is a polypeptide substance and is the first incretin analogue, a diabetes medication prepared by synthesizing exendin-4, a salivary substance of Gila monster. Exendin-4 is different from exendin-3 for only #2 and #3 sites, is known to have a longer half-life than glucagon like peptide-1 (GLP-1) which is a diabetes medication having a half-lire shorter than two minutes for DPP-IV, an enzyme that is resistant for directly degrading incretin enzyme that is produced in mammals' stomachs after ingestion by DPP-IV (dipeptidyl peptidase-4) to serve beneficial roles of promoting insulin secretion and lowering blood sugar level, and also, it shows 2-4 hours of half-life in vivo experiment, and it has been confirmed that it can reach enough blood concentration with 2-3 times of intraperitoneal injection per day.

[0008] Also, exendin-4 is known to control gastrointestinal tracts' motility, reduces food intake and suppresses blood plasma glucagon, and recently PLGA microsphere type synthetic exendin-4 (product name: Byetta) has been authorized by US FDA and is about to be released. However, since this Byetta LAR product has complicated preparation process and is short in vivo half-life for exendin-4, which is about 4-6 hours, frequent administration of high dose exendin-4 is required, and the problem of medication release control based on quick excretion due to the low molecular weight of lower than 4200, and problems such as immunogenicity still exist.

[0009] Therefore, while studying a method to reduce administration frequency of exendin-4 and solve the low molecular weight problem of exendin-4, the inventors have completed the present invention after having confirmed the fact that it is possible to increase the production yield of PEGylated exendin-4 and treatment effect of medications by performing selective PEGylation via insertion of cysteine (Cys) amino acid into the site (#40 site) next to #39 site of C-terminal of exendin-4.

DISCLOSURE OF THE INVENTION

TECHNICAL PROBLEM

[0010] One object of the present invention is to provide an exendin-4 analogue in which a cysteine (Cys) is introduced into #40 site of C-terminal and is PEGylated with polyethylene glycol (PEG) or derivatives thereof.

[0011] Another object of the present invention is to provide a method of preparing the exendin-4 analogue.

[0012] Still another object of the present invention is to provide a pharmaceutical composition for prevention or treatment of diseases caused by insulin hypersecretion, containing the exendin-4 analogue as an active ingredient.

TECHNICAL SOLUTION

[0013] In order to achieve the objects, the present invention provides an exendin-4 analogue according to Claim 1.

[0014] The present invention also provides a method of preparing the exendin-4 analogue according to claim 10. Furthermore, the present invention provides a pharmaceutical composition for use in the prevention or treatment of diseases caused by insulin hypersecretion containing the exendin-4 analogue as an active ingredient.

ADVANTAGEOUS EFFECTS

[0015] By performing selective PEGylation according to the invention, the yield of an exendin-4 analogue in which a cysteine (Cys) is introduced into #40 site of the C-terminal and is PEGylated with polyethylene glycol (PEG) or derivatives thereof, can be increased, and treatment effect of medications can be increased, and thus the exendin-4 analogue can be beneficially used as a composition for prevention or treatment of diseases caused by insulin hypersecretion.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016]

FIG. 1 is a schematic view illustrating PEGylation of exendin-4 in which cysteine (Cys 40) is introduced into the C-terminal of example 1 of the present invention.

FIG. 2 is a schematic view illustrating PEGylation for lycine amine of exendin-4 of comparative example 1 of the present invention.

FIG. 3 is a schematic view illustrating PEGylation for N-terminal of exendin-4 of comparative example 2 of the present invention.

FIG. 4 is a view illustrating light absorbance of example 1 of the present invention.

FIG. 5 is a view illustrating light absorbance of comparative examples 1a to 1c of the present invention.

FIG. 6 is a view illustrating light absorbance of comparative example 2 of the present invention.

FIG. 7 is a view illustrating the production yield of example 1 of the present invention.

FIG. 8 is a drawing illustrating product yield of comparative examples 1a to 1c of the present invention.

FIG. 9 is a view illustrating the production yield of comparative example 2 of the present invention.

FIG. 10 is a view illustrating the affinity of a PEG bound exendin-4 analogue to a GLP-1 receptor according to an example of the present invention.

FIG. 11 is a schematic view illustrating PEG bound exendin-4 analogues of examples 4 and 5 of the present invention.

FIG. 12 is a view illustrating blood glucose level for diabetic mice administrated with a PEG bound exendin-4 analogue according to an example of the present invention.

MODE FOR CARRYING OUT THE INVENTION

[0017] Hereinafter, the present invention will be described in detail.

[0018] The present invention provides an exendin-4 analogue according to Claim 1.

[0019] The molecular weight of polyethylene glycol or a derivative thereof according to the present invention is 5-60 kDa, and preferably 20-50 kDa.

[0020] Specifically, the polyethylene glycol derivative is selected from methoxypolyethylene glycol succinimidylpropionate, methoxypolyethylene glycol N-hydroxysuccinimide, methoxypolyethylene glycol propionaldehyde, methoxypolyethylene glycol maleimide. Preferably, the polyethylene glycol derivative is trimeric methoxypolyethylene glycol maleimide.

[0021] Also, the present invention provides a method of preparing an exendin-4 analogue PEGylated with the polyethylene glycol or a derivative thereof, which includes a process of dissolving exendin-4 in which cysteine is introduced into #40 site of the C-terminal, and polyethylene glycol or a derivative thereof in phosphate buffer saline solution and reacting them at room temperature according to the claims.

[0022] Specifically, an exendin-4 analogue PEGylated with polyethylene glycol or a derivative thereof may be prepared by adding exendin-4 in which cysteine is introduced into #40 site of the C-terminal, and polyethylene glycol or a derivative thereof in a phosphate buffer saline solution in a mole ratio of 1:1-3 in a phosphate buffer saline having a pH range of 7.2-7.8, preferably pH 7.5, dissolving these ingredients, and perform a reaction for 1-3 hours at room temperature and performing a column chromatography after the reaction is completed.

[0023] When the phosphate buffer saline is not within the pH range, the yield may decrease. After the exendin-4 analogue PEGylated with polyethylene glycol or the derivative thereof is prepared, the molecular structure of the exendin-4 analogue may be confirmed by a mass spectroscope, a liquid chromatography, an X-ray diffraction analysis, a polarimetry, and comparison between calculated values and measured values of representative elements constituting the exendin-4 analogue.

[0024] Also, the present invention provides a pharmaceutical composition for use in the prevention or treatment of diseases caused by insulin hypersecretion, containing the exendin-4 analogue as an active ingredient.

[0025] The diseases caused by insulin hypersecretion may include Type 1 diabetes, Type 2 diabetes and diabetes complications.

[0026] As a result of having measured affinity to a GLP-1 receptor of exendin-4 analogue PEGylated with polyethylene glycol or a derivative thereof according to the present invention, IC50 value was 1.04

nM, and this was confirmed to show 120 times more activity than compound of example 1 (Nter-PEG-Ex4) (IC50 = 121. 78 nM) (refer to experimental example 1, Table 3 and FIG 10).

[0027] Also, for better understanding, a schematic diagram of the present invention's exendin-4 bound with a trimeric PEG at C40 site is shown in FIG. 11.

[0028] When the molecular weight of the bound PEG is 23K, PEG of 3KD is used as a PEG spacer, and PEG having 10KD molecular weight are bound to terminal of the 3KD (example 4). Also, similar to this, when the molecular weight of the bound PEG is 50, PEG of 10KD is used as a PEG spacer, and PEG having the molecular weight of 20KD are bound to terminal of the 10KD (example 5). At this time, as a result of having measured the required time of blood glucose level raising back to 8.35 mmol/L after having injected the exendin-4 of example 4 (C40-PEG23K-Ex4) and example 5 (C40-PEG50K-Ex4), low blood glucose level maintained from 45.5-56.1 hours after the administration of the medication (refer to experimental example 2, Table 4 and FIG. 12), which was confirmed to be more than twice of C40-PEG20K-Ex4 (23.2 hours) and control group (7.3 hours), enabling 7-8 times more stable maintenance of blood glucose level.

[0029] Therefore, the C40 site specific PEG bound exendin-4 compound according to the present invention can solve the drawback of quick excretion of medications due to the low molecular weight of existing exendin-4, has excellent affinity to the GLP-1 receptor, and has strong low blood glucose maintaining ability capable of maintaining blood glucose level up to 3-4 days after having administrated the medications, so it can be used beneficially for preventing or treating insulin hypersecretion related Type 1 diabetes, Type 2 diabetes and diseases related with diabetes complications.

[0030] When the composition of the present invention is used as medications, the pharmaceutical composition containing the exendin-4 analogue PEGylated with polyethylene glycol or a derivative thereof may be administrated after having formulated into various oral or non-oral administration forms as the following in case of clinical administration. For oral administration purposed formulation, for example, there are tablets, pellets, hard/soft capsules, liquids, suspensions, emulsifiers, syrups, granules, elixirs, troches, and these formulations include diluents (example: lactose, dextrose, sucrose, mannitol, sorbitol, cellulose and/or glycine), slip modifiers (example: silica, talc, stearate and its magnesium or calcium salt and/or polyethylene glycol) in addition to the active ingredient. Tablets may also include binders such as magnesium aluminum silicate, starch paste, gelatin, methyl cellulose, sodium carboxymethyl cellulose and/or polyvinyl pyrrolidine, and may include disintegrating agents such as starch, agar, alginic acid or sodium salt thereof or boiling mixture and/or absorbents, coloring agents, flavoring agents and sweetening agents if needed.

[0031] The pharmaceutical composition containing the exendin-4 analogue PEGylated with polyethylene glycol or a derivative thereof may be non-orally administrated, and the administration is done by subcutaneous injection, intravenous injection, intramuscular injection or intrathoracic injection.

[0032] At this time, the exendin-4 analogue PEGylated with polyethylene glycol or a derivative thereof may be may be prepared into liquid or suspension by having mixed it with stabilizer or buffer in water to formulize it into non-orally administration purposed formulation, and this may be prepared into ampoule or vial unit administration form. The composition is sterilized and/or may include adjuvants such as antiseptics, stabilizers, hydrators or emulsify stimulators, osmotic pressure controlling purposed salts and/or buffers, and other substances beneficial for treatments, and may be formulated according to traditional methods of mixture, granulation or coating.

[0033] The human body dose of the pharmaceutical composition containing the exendin-4 analogue PEGylated with polyethylene glycol or a derivative thereof according to the present invention may vary depending on the age, body weight, gender, administration form, health status and level of disease of patients, and may be administrated via oral or non-oral route following decisions of doctors or pharmacists with preferably dose of 0.01 to 200 mg/kg/day.

MODE FOR CARRYING OUT THE INVENTION

[0034] The present invention will be explained in detail by examples and experimental examples hereafter.

Examples 1-5 (Examples 1-3 represent reference examples): C40 site specific PEG bound exendin-4 production

[0035] To prepare C40 site specific PEG bound exendin-4, exendin-4-Cys in which cysteine is introduced into the C-terminal site (#40 site) was used (exendin-Cys, molecular weight: 4290.7, sequence: SEQ ID NO:1:

HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPSC), and maleimide activated monomethoxy PEG (mPEG-MAL, MW: 5, 20 kDa(Linear type), 20 kDa (Branch type), 23, 50 kDa(Trimer type)) was purchased from Nippon Oil and Fats, NOF, Tokyo, and used.

[0036] To prepare C-terminal #40 site specific PEG bound exendin-4, exendin-4-Cys and mPEG-MAL (MW: 5, 20 (linear type), 20 (branch type), 23, 50 kDa) were completely dissolved in a mole ratio of 1:2 in a 20 mM phosphate buffer saline (pH 7.5) and were reacted for two hours at room temperature (refer to FIG. 1). After the reaction, the reacted solution was separated by a reversed phase chromatography with Capcell-pak RP-18 column (250 x 10 mm, 5 μm, Shiseido, Japan) at a flow speed of 5.0 ml/min. The separation was monitored at 215 nm wavelength ultraviolet ray. The mobile phase was separated using a linear concentration gradient method (36-42% B over 30 min) for 0.1% TFA distilled water (mobile phase A) and 0.1% TFA acetonitrile (mobile phase B) (refer to FIG. 4).

[0037] The peaks separated by the method were collected separately, acetonitrile was removed using nitrogen gas, and the removed solution was concentrated using Centricon-10 (Mw cut off 3000, Millipore Corp., Billerica, MA). The prepared substance was stored at 4 °C and prepared by mixing 1 μl sample-matrix sample solution and 2 μl matrix solution, and the matrix solution was prepared by dissolving α-cyanohydroxycinnamic acid (α-CHCA) with water/CAN (50:50) solution containing 0.1% (v/v) TFA. The prepared 1 μl sample-matrix solution was put on a sample plate, dried at vacuum status and analyzed with size exclusion chromatography (SEC) and MALDI-TOF mass spectrometer, and C40 site specific PEG bonding reaction (C40-PEG-Ex4) was analyzed at 0, 20, 40, 60 and 80 minutes and was shown with chromatogram area ratio in comparison with the initial status of exendin-4 and C40-PEG-Ex4. The result is illustrated in Table 1 and FIG. 7.

Table 1

	Reaction Time	Yield (%)
Example 1 C40-PEG_{5K}-Ex4 (linear)	80 min.	93%
Example 2 C40-PEG_{20K}-Ex4 (linear)	80 min.	89%
Example 3 C40-PEG_{20K}-Ex4 (branch)	80 min.	91%
Example 4 C40-PEG_{23K}-Ex4 (trimer)	80 min.	90%
Example 5 C40-PEG_{50K}-Ex4 (trimer)	80 min.	85%

[0038] As shown in Table 1, the reaction time was 80 minutes in average, production being done with yield of over 90% average (refer to FIG. 7).

Comparative Example 1: Production of non-specific PEG bound exendin-4

[0039] A method equivalent to the example 1 except for using exendin-4 (molecular weight: 4186.6, sequence: SEQ ID NO: 2 HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS) and succinimidyl activated monomethoxy PEG (mPEG-SPA, MW: 5, 20 kDa (Linear type)) instead of using cysteine introduced exendin-4-Cys and maleimide activated monomethoxy PEG, was performed to prepare non-specific PEG bound exendin-4 (refer to FIG. 2 and FIG. 5).

[0040] The succinimidyl activated monomethoxy PEG (mPEG-SPA) was purchased from Nippon Oil and Fats, NOF, Tokyo, and used.

Table 2

Comparative example 1	Reaction Time	Yield (%)
Comparative example 1a Lys^{12}-PEG_{20K}-Ex4	80 min.	20%
Comparative example 1b Lys^{27} -PEG_{20K}-Ex4	80 min.	31%
Comparative example 1c $Lys^{12,27}$-PEG_{20K}-Ex4	80 min.	25%

[0041] As shown in Table 2, the reaction time of non-specific primary amine PEG binding reaction was 80 minutes in average, average yield being 20% for Comparative example 1a(Lys^{12}-PEG_{20K}-Ex4) and 31% for Comparative example 1b(Lys^{27}-PEG_{20K}-Ex4) (refer to FIG. 8).

Comparative example 2: N-Terminal specific PEG bound exendin-4 production

[0042] A method equivalent to the example 1 except for using exendin-4 (molecular weight: 4186.6, sequence: HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS) and monomethoxy PEG-aldehyde (mPEG-ALD, MW: 5 kDa (linear)) instead of using cysteine introduced exendin-4-Cys and maleimide activated monomethoxy PEG, was performed to prepare non-specific PEG bound exendin-4 (refer to FIG. 3 and FIG. 6).

[0043] The monomethoxy PEG-aldehyde was purchased from Nippon Oil and Fats, NOF, Tokyo and used.

[0044] As a result, the reaction time of N-terminal specific PEG binding reaction (N_{ter}-PEG_{5K}-Ex4) was 720 minutes, with average yield of 72% (refer to FIG. 9).

Comparative Example 3 : Analysis of RIN-m5F cell receptor binding affinity of PEG bound exendin-4 analogue

[0045] The following experiment was performed to perform GLP-1 receptor (GLP-1R) affinity of PEG bound exendin-4 analogues of Example 1(C40-PEG_{5K}-Ex4), Comparative example 1a (Lys^{12}-PEG_{5K}-Ex4), Comparative example 1b (Lys^{27}-PEG_{5K}-Ex4) and Comparative example 2(N_{ter}-PEG_{5K}-Ex4) prepared in Example 1, Comparative example 1 and 2.

[0046] Islet cells (RIN-m5F, ATCC, Manassas, VA) expressing vast quantity of GLP-1 receptor (GLP-1R) were inoculated in 12-wells plates. It was washed twice with binding buffer (120 mM NaCl, 1.2 mM $MgSO_4$, 13 mM sodium acetate, 5 mM KCl, 1.2 g/ℓ Tris, 2 g/ℓ bovine serum albumin, 1.8 g/ℓ glucose, pH 7.6) after 48 hours and unmarked PEG bound exendin-4 analogue (final concentration range: 0.001-1000 nM) and exendin-4 marked with 30 pM concentration I-125 (9-39, PerkinElmer, Boston, MA) were treated simultaneously. Thorough washing was done with PBS including 1 mg/mℓ bovine serum albumin after two hours. Finally the cells were thoroughly degraded for 15 minutes using cell lysis buffer (0.5 N NaOH with 1% SDS), and the radiation level of I-125 was measured using a gamma counter (GMI, Inc., Ramsey, MN). The result is illustrated in Table 3 and FIG. 10.

Table 3

	IC_{50}(nM)
Example 1 (C40-PEG_{5K}-Ex4)	1.04 nM
Comparative example 1a (Lys^{12}-PEG_{5K}-Ex4)	6.45 nM
Comparative example 1b (Lys^{27}-PEG_{5K}-Ex4)	2.42 nM
Comparative example 2 (N_{ter}-PEG_{5K}-EX4)	121.78 nM

	IC_{50}(nM)
Control group (exendin-4)	0.23 nM

[0047] As shown in Table 3, IC_{50} of Example 1(C40-PEG_{5K}-Ex4) according to the present invention was confirmed to be 1. 04 nM after the affinity for GLP-1 receptor was measured. It was confirmed that it shows activity twice better than Comparative example 1b (Lys^{27}-PEG_{5K}-Ex4) (IC_{50}value = 2.42 nM), and 6 times better than Comparative example 1a (Lys^{12}-PEG_{5K}-Ex4) (IC_{50} value= 6.45 nM). Also, it was confirmed that Example 1(C40-PEG_{5K}-Ex4) according to the present invention shows activity 120 times better than Comparative example 2(N_{ter}-PEG_{5K}-Ex4) (IC_{50}value= 121.78 nM).

[0048] Therefore, C40 site specific PEG bound exendin-4 composition according to the present invention not only can solve the weakness of quick excretion of medications due to low molecular weight of exendin-4, but also can be used beneficially as a diabetes medication since GLP-1 receptor affinity shows similar biological activity as exendin-4 (refer to FIG. 10).

Experimental Example 1: Evaluation of low blood glucose sustainability in non-fasting Type 2 diabetic mice

[0049] The following experiment was performed to evaluate low blood glucose sustainability of C40 site specific PEG bound exendin-4 composition according to the present invention in Type 2 diabetic mice.

[0050] Type 2 diabetic C57BL/6 db/db mice (male, 4-5 weeks old, Central Lab. Animal Inc.) were used, and animals were exposed to light at 12 hours cycle and were grown after having stabilized two week by allowing free intake of foods and water. The experimental animals were managed following the guideline of National Institute of Health (NIH) and authorized by Institutional Animal Care and Use Committee of Sungkyunkwan University, and the experiment was performed humanely.

[0051] C40-PEG_{5K}-Ex4 (linear), C40-PEG_{20K}-Ex4 (linear), C40-PEG_{20K}-Ex4 (branch), C40-PEG_{23K}-Ex4 (trimer) and C40-PEG_{50K}-Ex4 (trimer) prepared from the Example 1 to 5 and Lys^{27}-PEG_{20K}-Ex4 prepared in Comparative example 1bwere intraperitoneally injected with 25 nmol/kg dose to male db/db mice (6-7 weeks old), blood was collected from tail vein of mice following the float time:0, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36, 48, 60, 72, 96 hours and blood glucose concentration was measured with ACCU-CHEK Sensor (Roche Diagnostics Corp., USA). Afterwards, the low blood glucose sustaining time (blood glucose level < 8.35 mmol/ℓ (150 mg/dL)) was additionally measured and shown in Table 4 and FIG. 12. In the present experiment, exendin-4 was used as the control group.

Table 4

	Blood glucose level (mmol/l) (average)							
	C40-PEG-Ex4					Comparative Example 1b (Lys^{27}-PEG_{20K}-Ex4)	Control group (Ex-4)	Untreated group
Time (h)	Example 1 (PEG_{5K})	Example 2 (PEG_{20K})	Example 3 (PEG_{20K})	Example 4 (PEG_{23K})	Example 5 (PEG_{50K})			
0	23.38	24.28	24.44	24.22	24.56	24.13	22.61	24.23

Time (h)	Blood glucose level (mmol/l) (average)							
	C40-PEG-Ex4					Comparative Example 1b (Lys^{27}-PEG_{20K}-Ex4)	Control group (Ex-4)	Untreated group
	Example 1 (PEG_{5K})	Example 2 (PEG_{20K})	Example 3 (PEG_{20K})	Example 4 (PEG_{23K})	Example 5 (PEG_{50K})			
0.5	7.62	7.96	7.86	7.97	7.63	7.97	6.95	24.21
1	7.36	6.13	6.89	6.99	6.25	6.56	6.41	23.44
2	5.09	5.29	5.04	4.96	5.45	5.24	5.80	24.58
3	4.46	4.18	4.15	4.11	4.64	4.22	5.85	22.96
4	4.93	4.34	4.66	4.54	4.23	4.29	8.02	24.54
6	5.73	4.9	4.67	4.87	4.26	4.85	10.69	23.43
8	9.04	4.57	5.11	4.66	4.69	5.13	16.01	24.94
12	16.2	5.86	7.89	4.9	4.87	5.40	23.89	22.47
24	21.1	8.54	15.09	5.52	5.11	12.98	-	24.42
36	-	11.47	20.14	8.08	6.31	17.34	-	23.92
48	-	15.34	24.21	8.66	7.26	20.45	-	22.66
60	-	20.45	23.76	11.34	8.87	23.02	-	23.41
72	-	23.02	-	14.12	13.49	-	-	22.26
96	-	-	-	18.79	17.07	-	-	24.51
120	-	-	-	24.53	23.02	-	-	23.75

[0052] As shown in Table 4, the time required for blood glucose level of C40 site specific PEG bound exendin-4 of Examples 1 to 5 according to the present invention increasing back to 8.35 mmol/ℓ was confirmed to be longer than exendin-4 (7.3 hours), and especially for Example 4(C40-PEG_{23K}-Ex4) and Example 5(C40-PEG_{50K}-Ex4) which were introduced with trimer PEG, the low blood glucose level sustained for 45.5 hours and 56.1 hours, respectively (refer to FIG. 12).

[0053] Therefore, C40 site specific PEG bound exendin-4 composition according to the present invention can be used beneficially as a diabetes medication by having solved the weakness of quick excretion of medications due to low molecular weight of exendin-4 and consequently sustaining blood glucose level 7-8 times more stable than the Comparative examples.

[0054] Meanwhile, C40 site specific PEG bound exendin-4 analogue according to the present invention can be formulated into various forms following purposes. The following is an illustration of few formulation methods that include C40 site specific PEG bound exendin-4 analogue according to the present invention as an active ingredient, and the present invention is not limited thereof.

Formulation example 1: Production of powders

[0055] C40 site specific PEG bound exendin-4 analogue 2 g
Lactose 1 g

The ingredients were mixed and stuffed in sealed packages to prepare powders.

Formulation example 2: Production of tablets

[0056] C40 site specific PEG bound exendin-4 analogue 100 mg
Corn starch 100 mg
Lactose 100 mg
Magnesium stearate 2 mg
The ingredients were mixed and compressed according to general preparation methods for tablets to prepare tablets.

Formulation example 3: Production of capsule

[0057] C40 site specific PEG bound exendin-4 analogue 100 mg
Corn starch 100 mg
Lactose 100 mg
Magnesium stearate 2 mg
The ingredients were mixed and stuffed in gelatin capsules according to general preparation methods for capsules to prepare capsules.

Formulation example 4: Production of injections

[0058] C40 site specific PEG bound exendin-4 analogue 100 mg
Mannitol 180 mg
$Na_2HPO_4 \cdot 2H_2O$ 26 mg
Distilled water 2974 mg
The ingredients were included with the given quantity according to general preparation methods for injections to prepare injections.

INDUSTRIAL APPLICABILITY

[0059] By performing selective PEGylation according to the invention, the yield of an exendin-4 analogue in which a cysteine (Cys) is introduced into #40 site of the C-terminal and is PEGylated with polyethylene glycol (PEG) or a derivative thereof, can be increased and the treatment effect of medications can be increased, and thus the exendin-4 analogue can be beneficially used as a composition for prevention or treatment of diseases caused by insulin hypersecretion.

SEQUENCE LISTING

[0060]

<110> B&L Delipharm, Corp.

<120> Exendin-4 analogue pegylated with polyethylene glycol or derivative thereof, preparation method thereof, and pharmaceutical composition for preventing or treating diabetes, containing same as active ingredient

<130> PABBF/P54793EP

<140> 12804683.6
<141> 2012-06-28

<150> KR-10-2011-0062858
<151> 2011-06-28

<150> PCT/KR2012/005137
<151> 2012-06-28

<160> 2

<170> BiSSAP 1.2

<210> 1
<211> 40
<212> PRT
<213> Artificial Sequence

<220>
<223> exendin-4 from Heloderma suspectum with an additional C at the carboxy terminus

<400> 1

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

<210> 2
<211> 39
<212> PRT
<213> Heloderma suspectum

<400> 2

```
His Gly Glu Gly Thr Phe Thr Ser Asp Leu Ser Lys Gln Met Glu Glu
1               5                  10                  15
Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser
            20                  25                  30
Ser Gly Ala Pro Pro Pro Ser
        35
```

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- WO2004022004A [0006]
- KR12804683 [0060]
- KR1020110062858 [0060]
- KR2012005137W [0060]

Patentkrav

1. Exendin-4-analog, hvor et cystein (Cys) er indført i #40-stedet af den C-terminale ende af exendin-4 og er PEGyleret med en trimerisk type af polyethylenglycol (PEG) eller et derivat deraf, hvor polyethylenglycolderivatet er udvalgt blandt methoxypolyethylenglycolsuccinimidylpropionat, methoxypolyethylenglycol-N-hydroxysuccinimid, methoxypolyethylenglycolpropionaldehyd, methoxypolyethylenglycolmaleimid.

2. Exendin-4-analog ifølge krav 1, hvor polyethylenglycol eller derivatet deraf har en molekylvægt på 5-60 kDa.

3. Exendin-4-analog ifølge krav 2, hvor polyethylenglycol eller derivatet deraf har en molekylvægt på 20-50 kDa.

4. Exendin-4-analog ifølge krav 1, hvor polyethylenglycolderivatet er trimerisk methoxypolyethylenglycolmaleimid.

5. Fremgangsmåde til fremstilling af exendin-4-analogen ifølge krav 1, hvilken fremgangsmåde omfatter: at opløse exendin-4, hvori et cystein er indført i #40-stedet af den C-terminale ende af exendin-4, og en trimerisk type af polyethylenglycol eller et derivat deraf i en i en phosphatbuffer-saltopløsning; og at omsætte de opløste bestanddele ved rumtemperatur, hvor polyethylenglycolderivatet er udvalgt blandt methoxypolyethylenglycolsuccinimidylpropionat, methoxypolyethylenglycol-N-hydroxysuccinimid, methoxypolyethylenglycolpropionaldehyd, methoxypolyethylenglycolmaleimid.

6. Fremgangsmåde ifølge krav 5, hvor phosphatbuffer-saltopløsningen har et pH-område på 7,2-7,8.

7. Fremgangsmåde ifølge krav 5, hvor reaktionsmolforholdet med exendin-4 med det indførte cystein og polyethylenglycol eller derivatet deraf er 1:1-3.

8. Farmaceutisk sammensætning til anvendelse til forebyggelse eller behandling af en sygdom forårsaget af hypersekretion af insulin, hvor sammensætningen omfatter en exendin-4-analog ifølge krav 1 som en aktiv bestanddel.

9. Farmaceutisk sammensætning ifølge krav 8 til anvendelse ifølge krav 8, hvor sygdommen forårsaget af hypersekretion af insulin er type 1-diabetes, type 2-diabetes eller diabeteskomplikationer.

DRAWINGS

Fig. 1

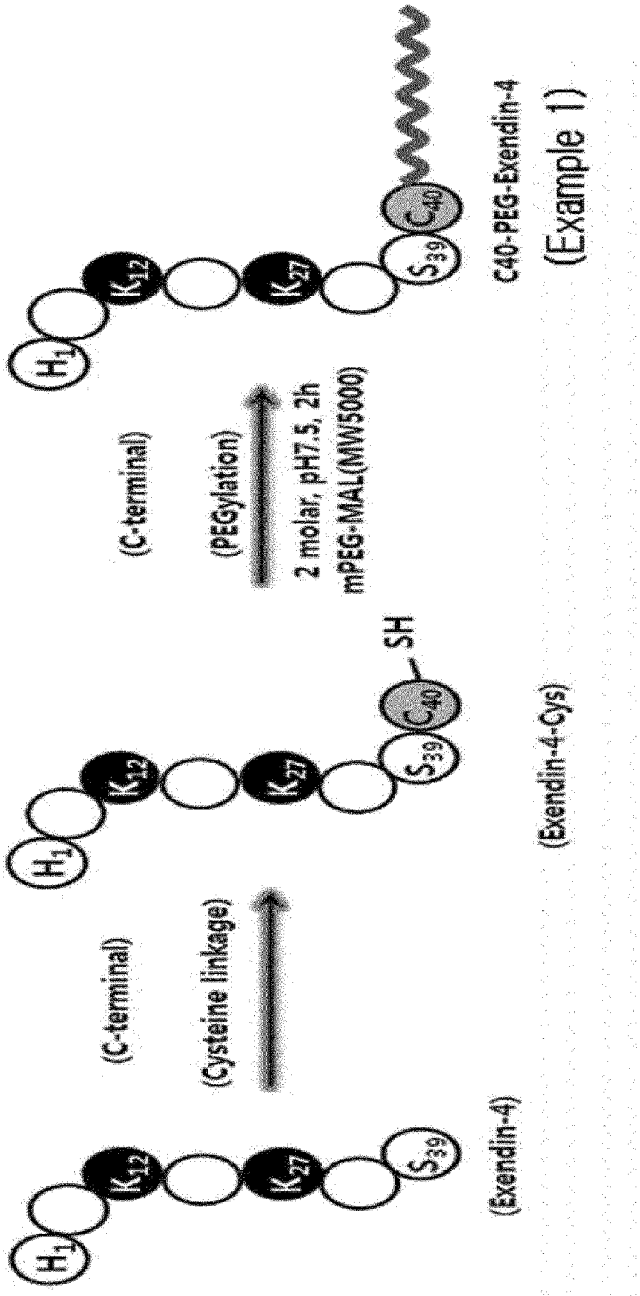


Fig. 2

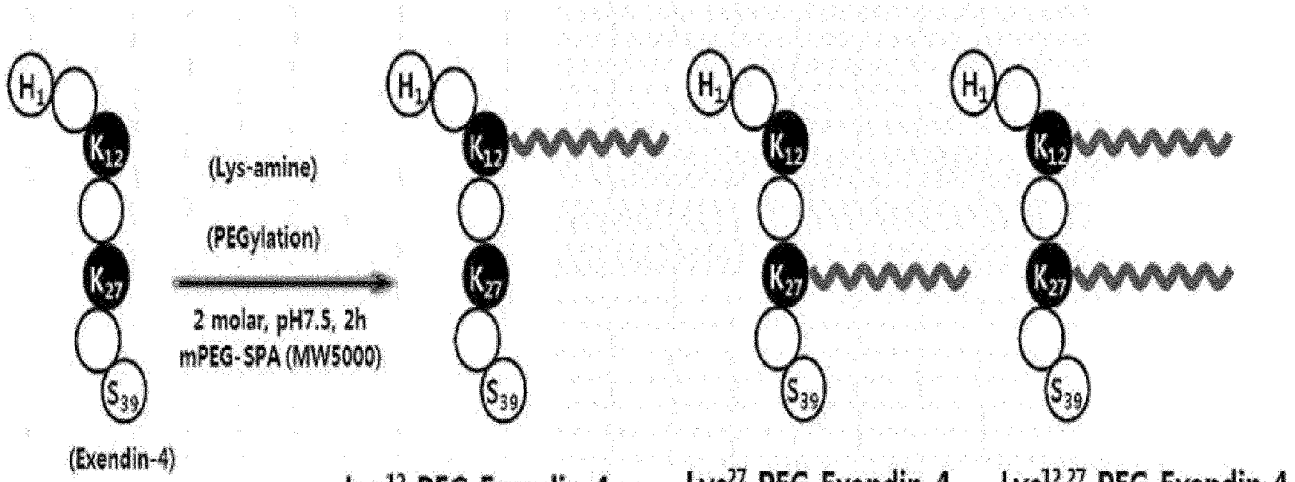


Fig. 3

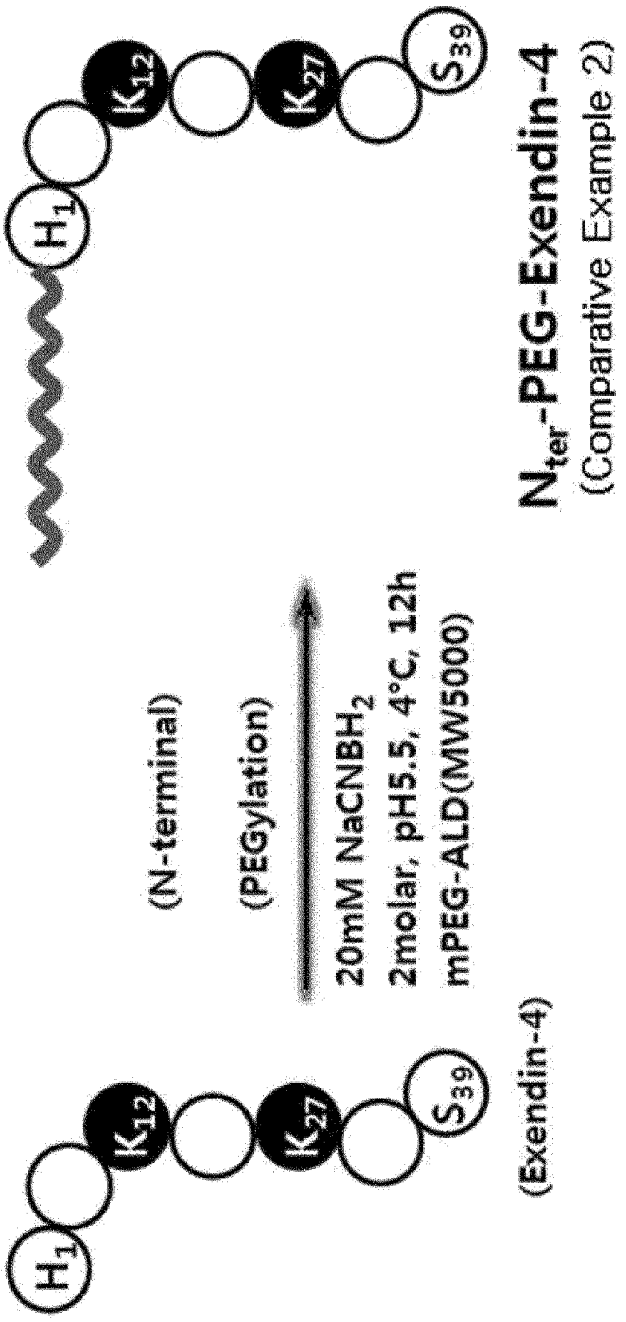

H1
K12
K27
S39
(Exendin-4)
(N-terminal)
(PEGylation)
20mM NaCNBH2
2molar, pH5.5, 4°C, 12h
mPEG-ALD(MW5000)
Nter-PEG-Exendin-4
(Comparative Example 2)

Fig. 4

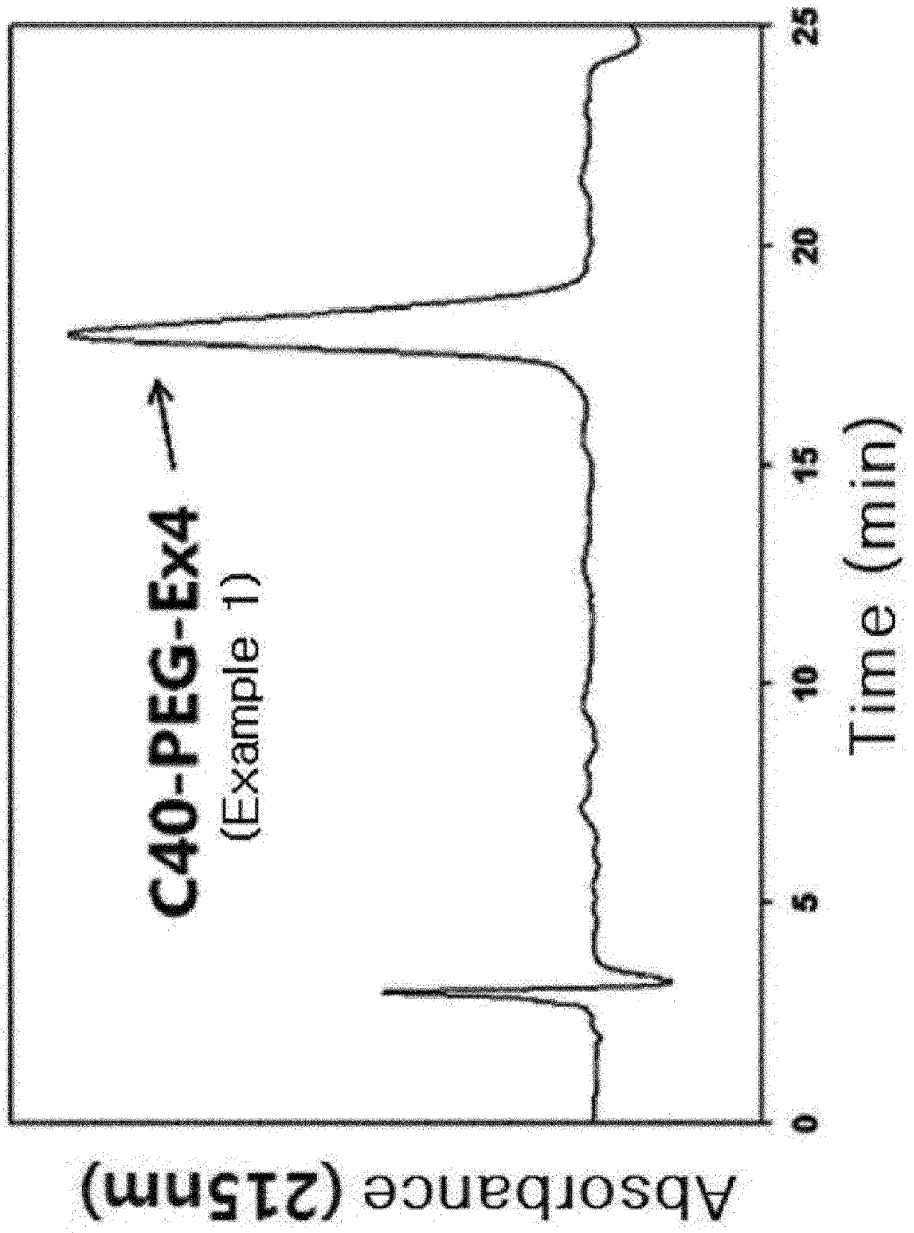


Fig. 5

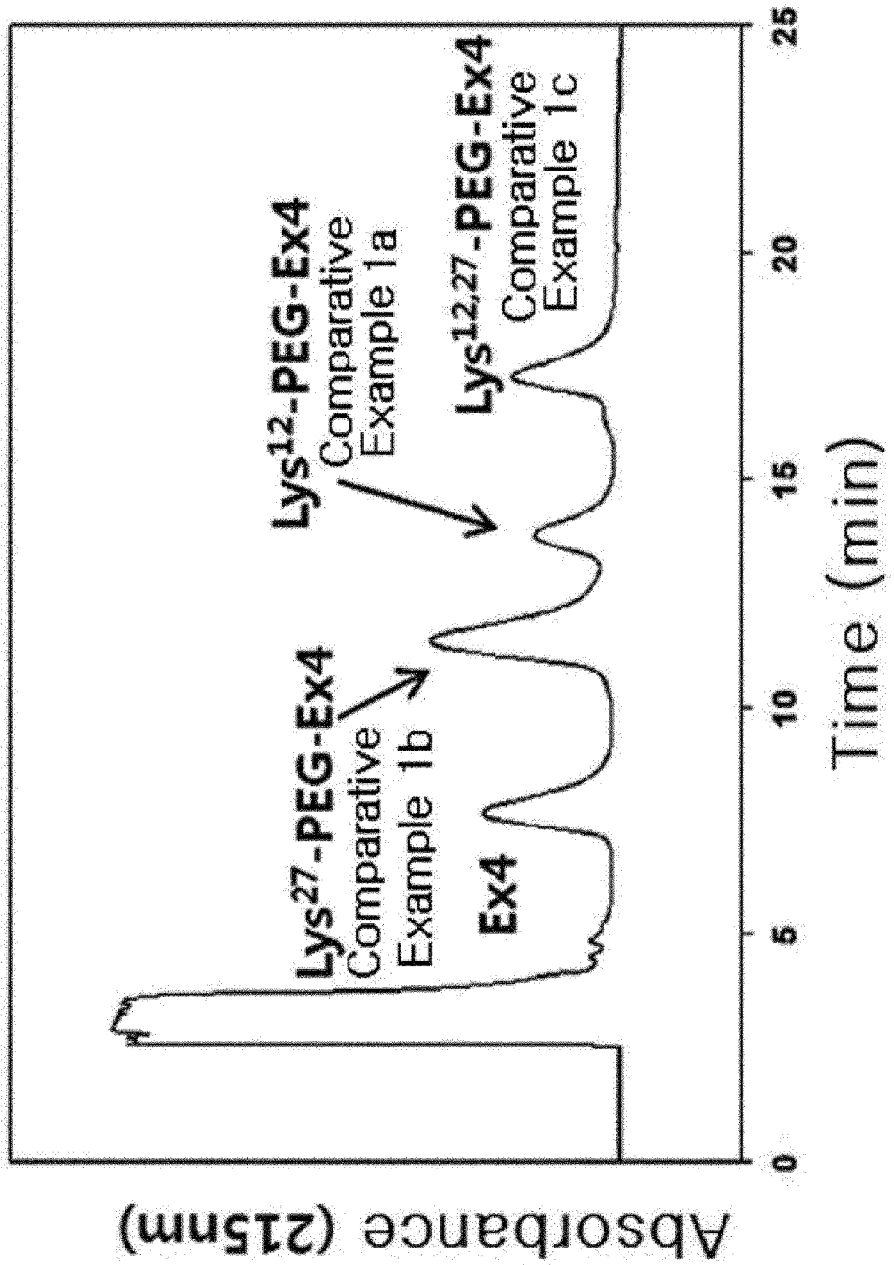

Absorbance (215nm)
Time (min)
0
5
10
15
20
25
Ex4
Lys27-PEG-Ex4
Comparative Example 1b
Lys12-PEG-Ex4
Comparative Example 1a
Lys12,27-PEG-Ex4
Comparative Example 1c

Fig. 6

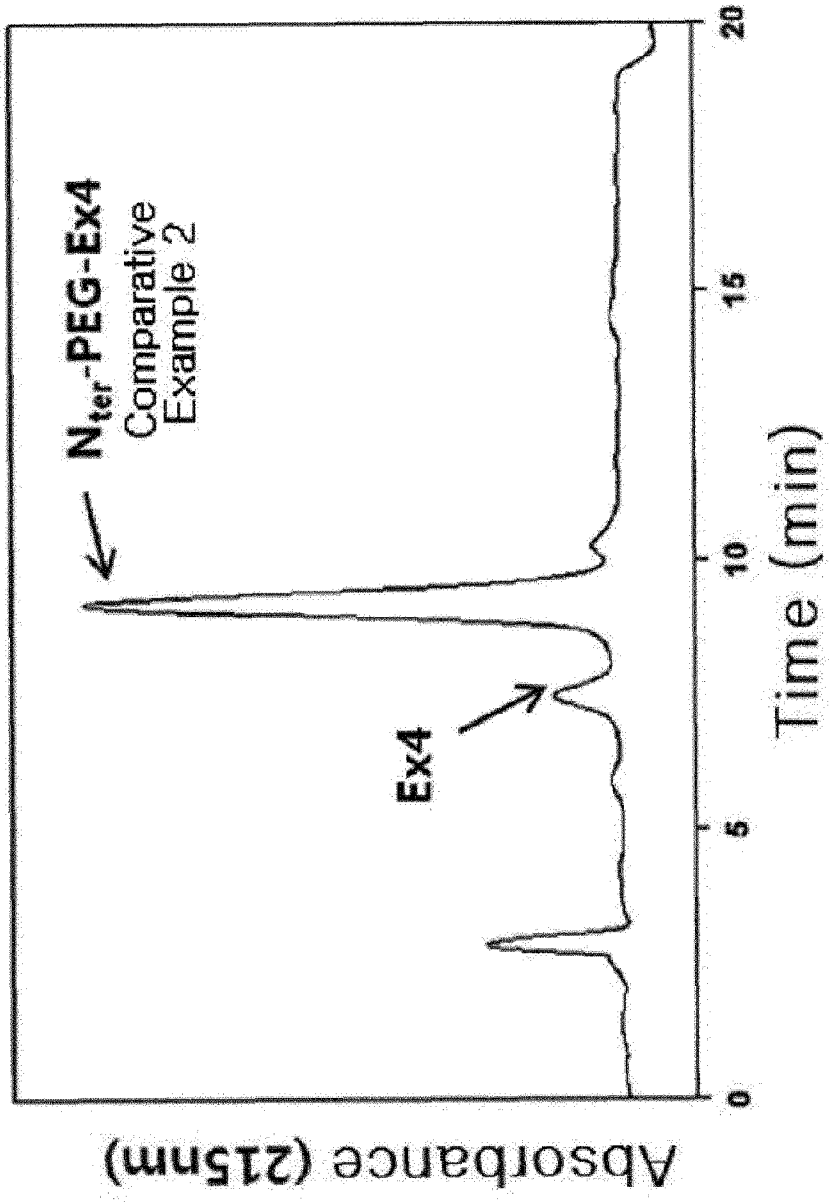

Absorbance (215nm)
Time (min)
0
5
10
15
20
Ex4
N_{ter}-PEG-Ex4
Comparative
Example 2

Fig. 7

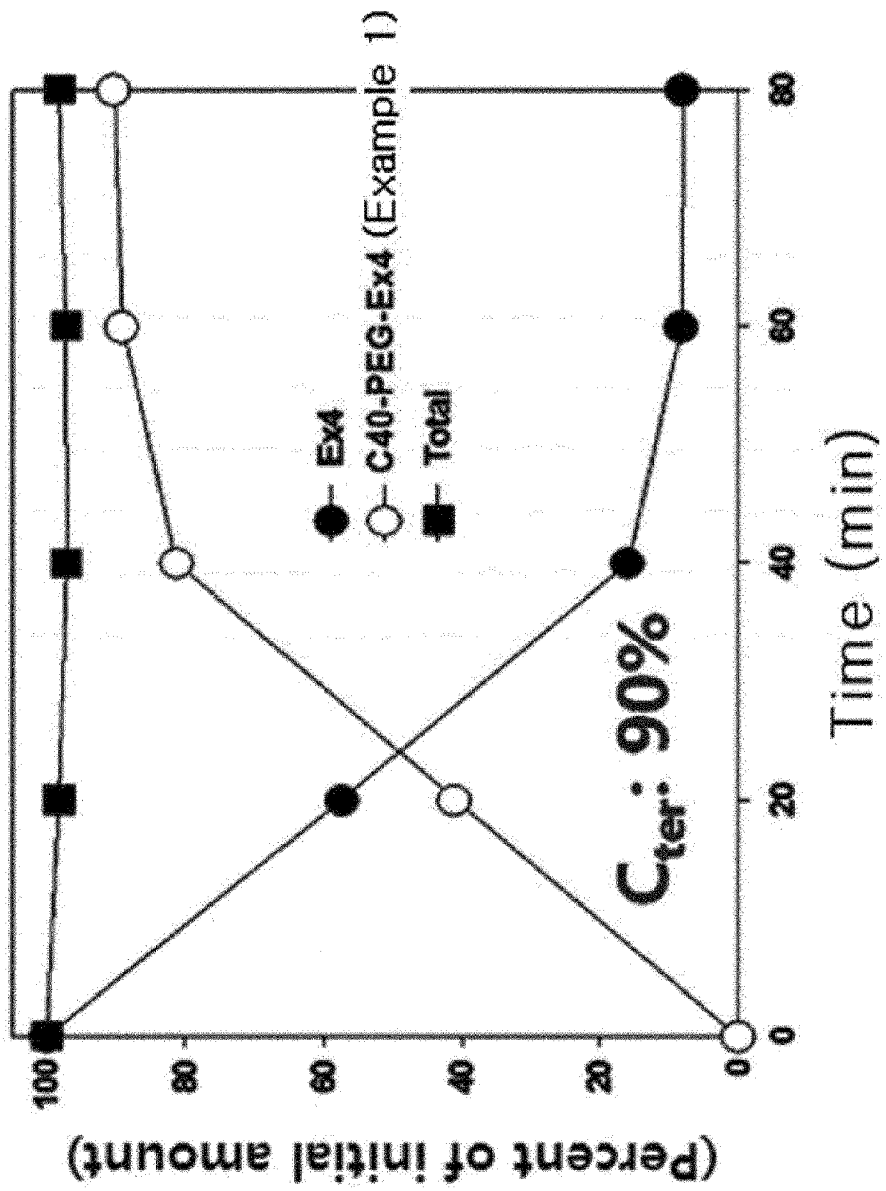

(Percent of initial amount)
100
80
60
40
20
0
0
20
40
60
80
Time (min)
Ex4
C40-PEG-Ex4 (Example 1)
Total
C_{ter}: 90%

Fig. 8

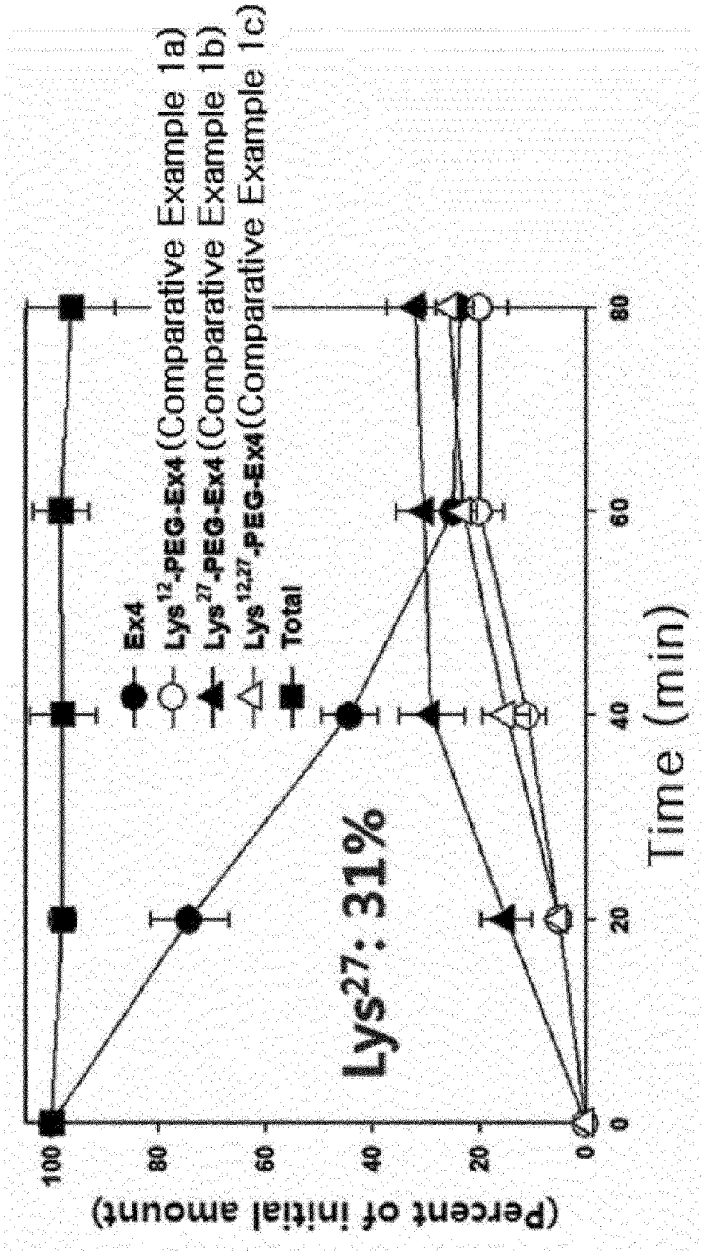

(Percent of initial amount)
Time (min)
Ex4
Lys12-PEG-Ex4 (Comparative Example 1a)
Lys27-PEG-Ex4 (Comparative Example 1b)
Lys12,27-PEG-Ex4 (Comparative Example 1c)
Total
Lys27: 31%
0
20
40
60
80
100

Fig. 9

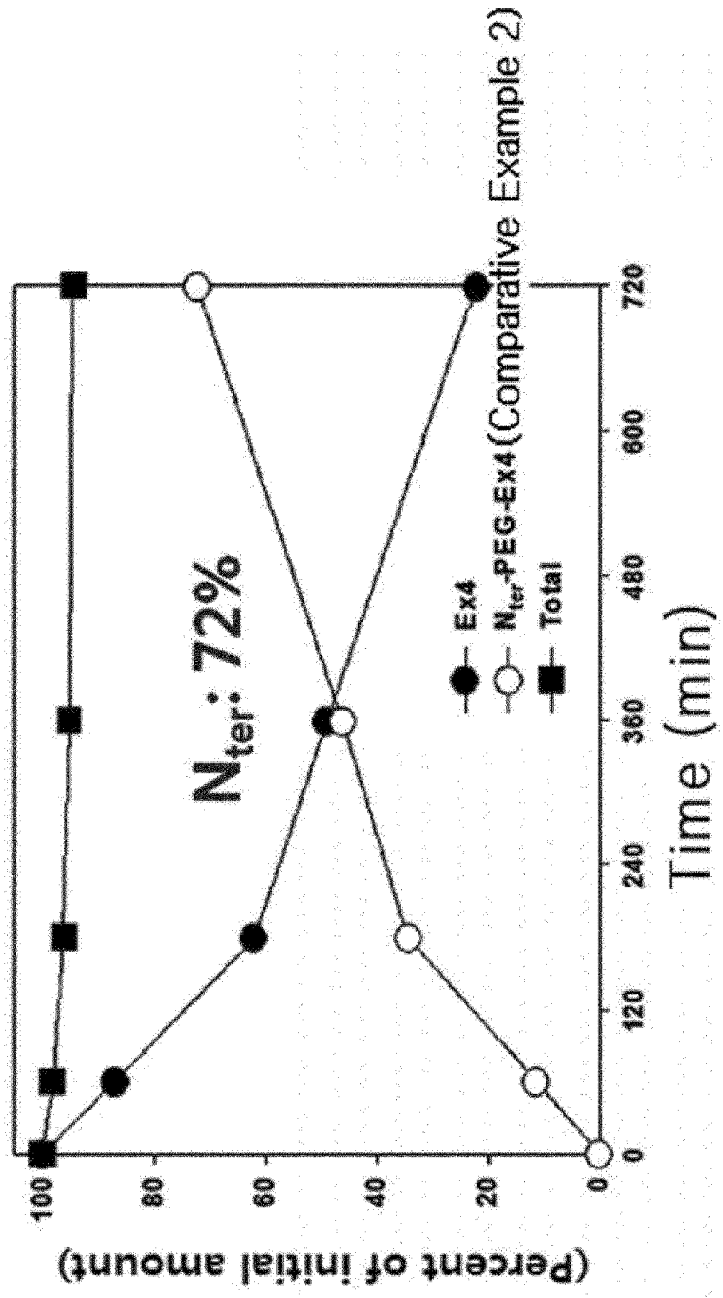

(Percent of initial amount)
100
80
60
40
20
0
0
120
240
360
480
600
720
Time (min)
Ex4
N_{ter}-PEG-Ex4 (Comparative Example 2)
Total
N_{ter}: 72%

Fig. 10

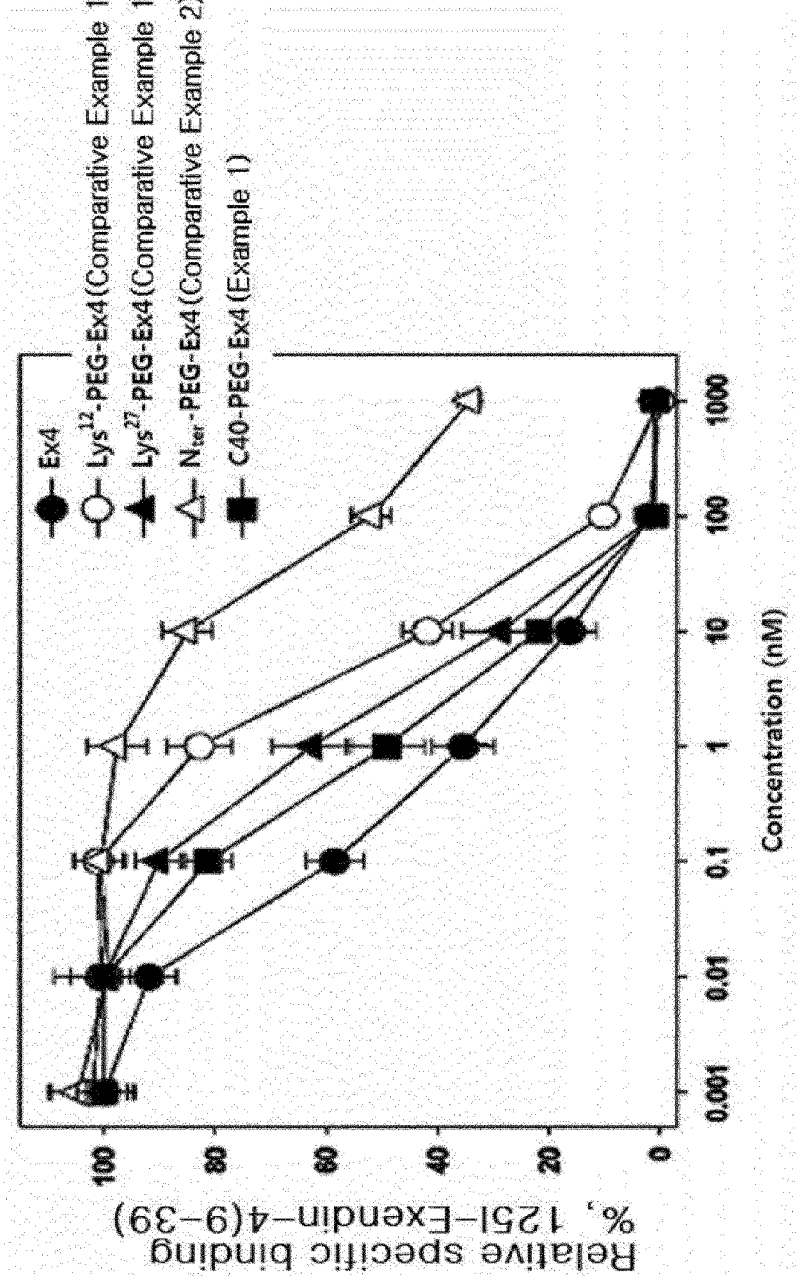

Ex4
Lys12-PEG-Ex4 (Comparative Example 1a)
Lys27-PEG-Ex4 (Comparative Example 1b)
Nter-PEG-Ex4 (Comparative Example 2)
C40-PEG-Ex4 (Example 1)
Relative specific binding
%, 125I-Exendin-4(9-39)
100
80
60
40
20
0
0.001
0.01
0.1
1
10
100
1000
Concentration (nM)

Fig. 11

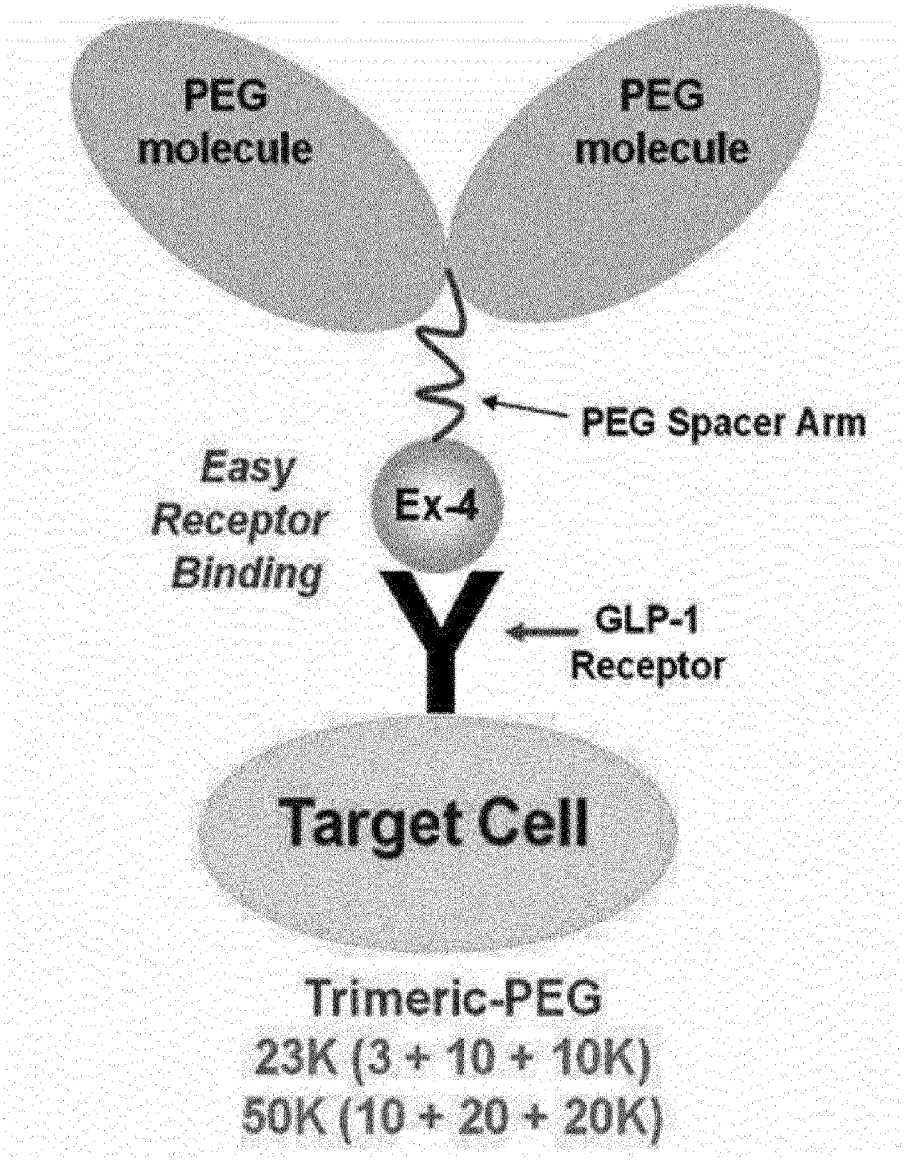
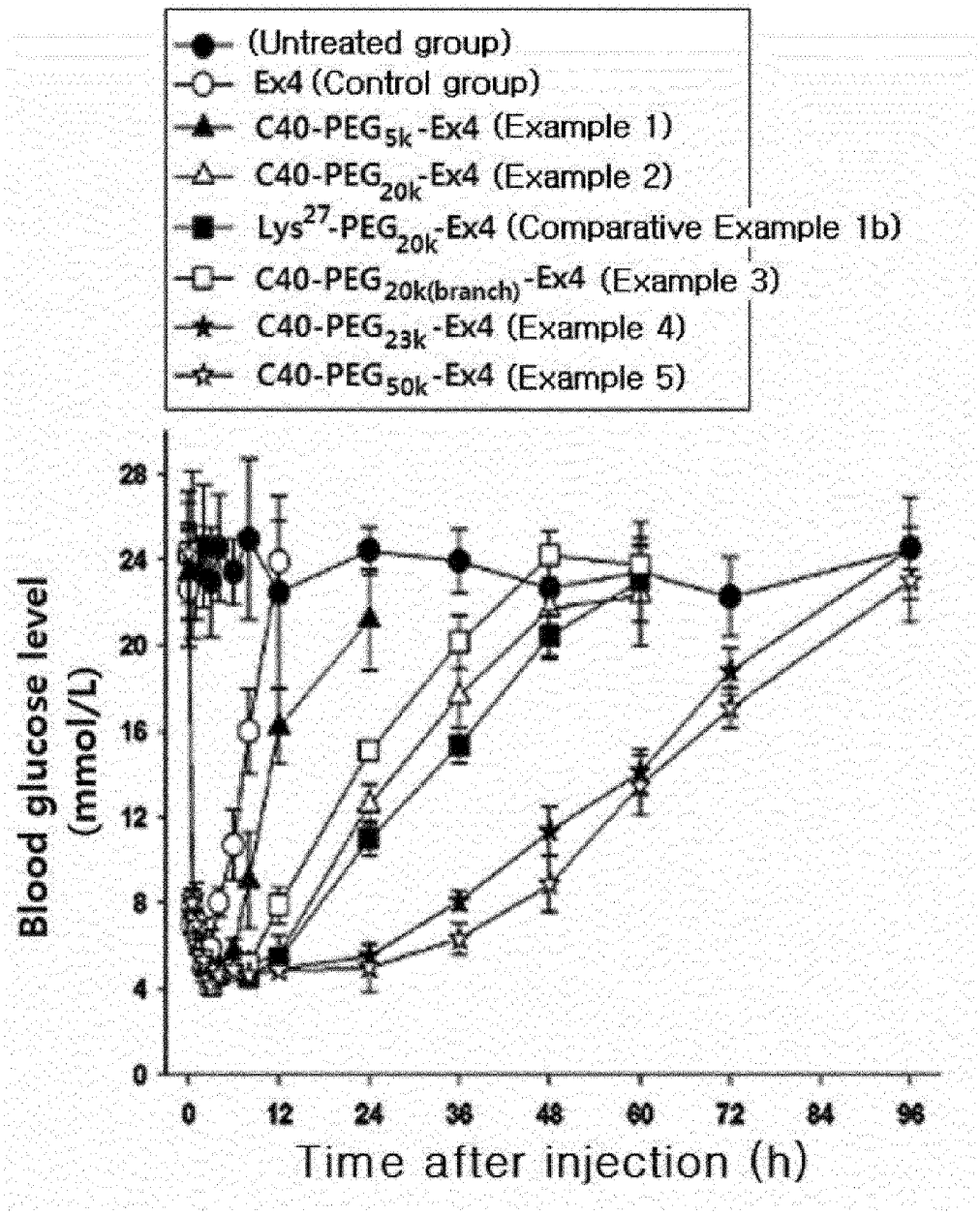

PEG molecule
PEG molecule
PEG Spacer Arm
Easy Receptor Binding
Ex-4
GLP-1 Receptor
Target Cell
Trimeric-PEG
23K (3 + 10 + 10K)
50K (10 + 20 + 20K)

Fig. 12


(Untreated group)
Ex4 (Control group)
C40-PEG5k-Ex4 (Example 1)
C40-PEG20k-Ex4 (Example 2)
Lys27-PEG20k-Ex4 (Comparative Example 1b)
C40-PEG20k(branch)-Ex4 (Example 3)
C40-PEG23k-Ex4 (Example 4)
C40-PEG50k-Ex4 (Example 5)
Blood glucose level (mmol/L)
0
4
8
12
16
20
24
28
0
12
24
36
48
60
72
84
96
Time after injection (h)