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(54) Title: AN IMPROVED PROCESS FOR PREPARATION OF PHYSIOLOGICALLY ACTIVE POLYPEPTIDE COMPLEX

(57) Abstract: Disclosed is a method for the preparation of a complex in which a physiologically active polypeptide is covalently bonded to an immunoglobulin constant region via a non-peptidyl linker. The method is characterized by the employment of a reducing agent, by which conventional problems of low production yield and modification of the polypeptide can be overcome. The physiologically active polypeptide-non-peptidyl polymer-immunoglobulin constant region complex can be produced with high purity and yield as well as at low cost. Thus, the method is industrially useful. Moreover, by exhibiting a prolonged action profile, the physiologically active polypeptide-non-peptidyl polymer-immunoglobulin constant region complex can be effectively used for developing long-acting formulations of physiologically active polypeptides which have improved drug compliance.

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Description

Title of Invention: AN IMPROVED PROCESS FOR PREPARATION OF PHYSIOLOGICALLY ACTIVE POLYPEPTIDE COMPLEX

Technical Field

- [1] The present invention relates to a method for the preparation of a complex in which a physiologically active polypeptide is covalently bonded to an immunoglobulin constant region via a non-peptidyl polymer linker comprising two or more aldehydes as functional groups. More particularly, the present invention relates to a method for effectively preparing a physiologically active polypeptide complex, characterized by improving problems of low production yield and reagent polypeptide deformation by adjusting the usage of a reducing agent when preparing the same.

[2]

Background Art

- [3] On the whole, physiologically active polypeptides are easy to denature due to their low stability, and readily undergo proteolytic degradation in blood and subsequent renal or hepatic clearance. Hence, protein drugs containing physiologically active polypeptides as pharmaceutical ingredients need to be frequently administered to patients in order to maintain appropriate serum levels and titers. However, such frequent administration of protein drugs, most of which is in the form of injections, causes pain to patients and has a high cost of treatment. To solve these problems, a lot of effort has been put into improving the serum stability of protein drugs and maintaining the drugs in the blood at high levels for a prolonged period of time to maximize the pharmaceutical efficacy of the drugs. As a requirement for use as long-acting preparations, protein drugs must be formulated to have high stability and have their titer maintained at a sufficiently high level without incurring immune responses in patients.
- [4] A conventional approach to stabilizing proteins and preventing enzymatic degradation and renal clearance is to chemically modify the surface of a protein drug with a polymer having high solubility, such as polyethylene glycol (hereinafter referred to as "PEG"). By binding to specific or various regions of a target protein, PEG makes the solubility of the protein higher, thereby stabilizing the protein and preventing hydrolysis without causing serious side effects (Sada et al., J. Fermentation Bio-engineering 71: 137-139).
- [5] For example, WO 2006/076471 discloses a way to make B-type natriuretic peptide (BNP) long-acting by conjugating it with PEGs which bind to the natriuretic peptide

receptor A (NPR-A) to trigger the synthesis of cGMP, thereby decreasing arterial blood pressure. It is thus used in the treatment of congestive heart failure. U.S. Patent No. 6,924,264 describes a method for improving the in vivo duration of exendin-4 by PEGylation of its lysine residue. However, in such a method, despite its capability to enhance the circulation time of the peptide drug by increasing the molecular weight of PEG, PEGylation has problems such as greatly reducing the titers of the peptide drug and decreasing yield due to the reduced reactivity of the peptide as the molecular weight of PEG increases.

- [6] WO 02/46227 discloses fusion proteins, in which GLP-1, and exendin-4 or analogs thereof are conjugated with human serum albumin or an immunoglobulin fragment (Fc) prepared by genetic recombination. U.S. Patent No. 6,756,480 concerns a fusion protein in which parathyroid hormone (PTH) or its analog is linked to an Fc. Although these methods may be evaluated as a solution to the problems of PEGylation, such as low yield and non-specificity, their effect in increasing the in vivo half-life of the target peptides has shown to not be significant, contrary to expectation, and further in some cases the fusion proteins have low titers. While a variety of peptidyl linkers are utilized so as to maximize the effect of increasing the serum half-life, they have a potential to evoke immune responses. Further, a peptide having a disulfide bond, such as BNP, has difficulty in practical application because it is highly apt to cause misfolding. Moreover, a peptidyl linker with non-natural amino acid residue is impossible to produce through genetic recombination.

- [7] Insulin is a peptide, secreted by beta cells of the pancreas in humans, and is central to regulating blood glucose level in the body. When insulin is not secreted properly or the secreted insulin does not adequately function within the body, blood glucose levels cannot be controlled and are increased, resulting in diabetes mellitus. The latter case is called type 2 diabetes. The case where insulin cannot be secreted from the pancreas and causes increased blood glucose levels, however, results in type 1 diabetes. Patients with type 2 diabetes are treated with oral hypoglycemic agents, and some of them are treated with insulin. Meanwhile, injection of insulin is essentially required for patients with type 1 diabetes.

- [8] Typically, insulin therapy is conducted by the administration of insulin via injection three times a day after or before every meal. However, continuous administration of insulin three times a day is painful and inconvenient for patients. Many attempts have been made to solve these problems. One strategy designed to increase the permeability of protein drugs in biomembranes is to deliver them by oral or nasal inhalation. However, administration by oral or nasal inhalation is significantly low in delivery efficiency when compared to injection, and has difficulty in maintaining peptide drugs at a level necessary for in vivo activity.

[9] As an alternative strategy, an excess amount of a drug can be subcutaneously injected and absorbed into the body in a delayed manner so as to maintain a constant blood level even with injection once a day. Some of the drugs (e.g., Lantus, Sanofi-aventis) are approved for commercial use and are currently administered to patients. Separately, a study has been conducted toward the modification of insulin with fatty acids to make the binding of insulin conjugates stronger and extend the duration through combining with albumin at the site of injection or in blood. Some of the drugs (e.g., Levemir, NovoNordisk) are approved for commercial use. However, these drugs cause pain at the site of injection and must be injected once a day, which is still a great burden to patients.

[10]

[11] To overcome the problems encountered in the prior art, the present inventors have prepared a complex comprising a physiologically active polypeptide and an immunoglobulin constant region that are connected by using a non-peptidyl polymer as a linker, as a strategy to simultaneously increase plasma half-life and in vivo duration of a physiologically active polypeptide such as insulin. However, there is also a need for a method for preparing the complex with a high yield and with high purity because the components of the complex are very expensive. With this in mind, the present inventors have developed a method to prepare the physiologically active polypeptide complexes with reduced cost, high yield and high purity by using use of proper kinds of reducing agents at an optimal concentration in a reaction solution during the reaction for complex production, thereby completing the present invention.

[12]

Disclosure of Invention

Technical Problem

[13] An object of the present invention is to provide an efficient method for preparing a complex in which a physiologically active polypeptide is covalently bonded to an immunoglobulin constant region via a non-peptidyl polymer linker comprising two or more aldehydes as functional groups, characterized by improving problems of low production yield and reagent polypeptide deformation by using proper kinds of reducing agents at the optimal concentration in a reaction solution during the reaction.

[14]

Solution to Problem

[15] In one aspect to achieve the above object, the present invention provides a method for preparing a complex of physiologically active polypeptide-non-peptidyl polymer-immunoglobulin constant region, comprising (1) reacting a non-peptidyl polymer having two or more aldehydes as functional groups with one of a physiologically

active polypeptide or an immunoglobulin constant region in the presence of a reducing agent at a concentration of 1 - 20 mM; and (2) reacting the reaction mixture of (1) with the other of a physiologically active polypeptide or immunoglobulin constant region in the presence of a reducing agent at a concentration of 1 - 100 mM.

- [16] The reaction mixture may comprise a conjugate of the non-peptidyl polymer and the physiologically active polypeptide or a conjugate of the non-peptidyl polymer and the immunoglobulin constant region, and/or the reactants that remain unreacted. Hence, the method of the present invention may further comprise separating the conjugate of the physiologically active polypeptide-non-peptidyl polymer or the conjugate of the immunoglobulin-non-peptidyl polymer from the reaction mixture after the step (1).
- [17] The term "non-peptidyl polymer," as used herein, refers to a biocompatible polymer comprised of at least two repeating units which are held together by a random covalent bond other than a peptide bond. Examples of the non-peptidyl polymer useful in the present invention include polyethylene glycols, polypropylene glycols, copolymers of ethylene glycol and propylene glycol, polyoxyethylated polyols, polyvinyl alcohols, polysaccharides, dextrans, polyvinyl ethyl ethers, biodegradable polymers such as polylactic acid (PLA) and polylactic-glycolic acid (PLGA), lipid polymers, chitins, hyaluronic acid and combinations thereof, with a preference for polyethylene glycol (PEG). The derivatives thereof that are well known in the art and derivatives which can be readily prepared using methods known in the art are also within the scope of the present invention.
- [18] As described above, the non-peptidyl polymer may have two or more aldehydes as functional groups. Thus, the non-peptidyl polymer listed above may be in a bi- or multi-functional aldehyde form in itself or contain a substituent having an aldehyde group at its two or more alcohol groups, preferably. The substituent having an aldehyde group may be alkylaldehydes such as propionaldehyde or butylaldehyde. In one preferred embodiment, the non-peptidyl polymer may be PEG with a propionaldehyde substituent at each of its termini.
- [19] The disadvantage of conventional peptidyl linkers used in fusion proteins constructed by an in-frame fusion technique is that they are readily cleaved in vivo by proteinases and thus cannot guarantee the prolongation of serum half-life by the carrier, contrary to expectation. However, in the present invention, a polymer that is resistant to protease is used, and thus the plasma half-life of the peptide can be maintained at a similar level to that of the carrier. Therefore, so long as it is resistant to in vivo proteinases, any non-peptidyl polymer may be used in the present invention, without limitation. Molecular weight of the non-peptidyl polymer ranges from 1 to 100 kDa and preferably from 1 to 20 kDa. In addition, the non-peptidyl polymer which is linked to the physiologically active polypeptide may be an individual polymer or a combination of different

polymers. Moreover, the non-peptidyl polymer useful in the present invention may have functional groups at its two or three ends which can be coupled to the physiologically active polypeptide and the immunoglobulin constant region. Preferably, the functional groups may be aldehyde.

- [20] Conjugation with PEG, which is typically used to prepare long-acting formulations of protein drugs, increases the stability of the proteins, while larger molecular weights of PEG exhibit lower reactivity with the proteins and thus decrease the production yield. Since the production yield is closely correlated with production cost and industrial applicability, it is very important to increase the production yield. PEG with aldehydes as functional groups may be coupled to an amine group, which is present at an N-terminus or on a side chain of Lys residue of the physiologically active polypeptide or the immunoglobulin constant region. In this regard, the yield of PEGylation may vary depending on various factors including the molar ratio of PEG to proteins, the concentration of reaction solutions, the time of reaction, pH, temperature, etc. Chem. Biol. Drug Des. 2007; 69; 132-138 describes insulin PEGylation carried out with 5 K aldehyde mPEG at a yield of over 90% by adjusting various factors including molar ratios, reaction times, pH, etc. In US 2009/0252703A1, it is reported that the addition of an organic solvent to the reaction solution increases the yield of peptide PEGylation. WO 2010/089756A2 discloses an improvement in the PEGylation yield by reacting r-metHuG-CSF with PEG in the presence of a carbohydrate.
- [21] However, when a non-peptidyl polymer including PEG with two or more functional groups is used as a linker between two different polypeptides, two or more steps in reactions are required, thus lowering the overall yield. Particularly, a step of the second reaction (wherein the physiologically active polypeptide or immunoglobulin constant region conjugated with a non-peptidyl polymer having two or more functional groups is reacted with the immunoglobulin constant region or the physiologically active polypeptide, respectively, hereinafter referred as "coupling reaction") was observed to be conducted with a significantly lower yield, compared to a step of the first reaction in which the physiologically active polypeptide or the immunoglobulin constant region is reacted with a non-peptidyl polymer having two or more functional groups.
- [22] In the present invention, the concentration of the reducing agent in the first reaction is demonstrated to have a correlation with the yield of the second coupling reaction. Higher yields of the coupling reaction were observed with lower concentrations of a reducing agent, shorter reaction times, and lower reaction temperatures in the first reaction.
- [23] As used herein, the term "reducing agent" refers to a compound that functions to reduce the reversible imine double formed from a reaction between the aldehyde group of the non-peptidyl polymer and the amine group of the polypeptides (physiologically

active polypeptide, immunoglobulin constant region), thereby forming a covalent bond and is intended to encompass all reducing agents known in the art. For the purpose of the present invention, the reducing agent may be added to a reaction solution in which the non-peptidyl polymer forms a covalent bond with the physiologically active polypeptide or the immunoglobulin constant region. So long as it is typically used in the art, any reducing agent may be employed in the present invention. Examples of the reducing agent may include, but are not limited to, sodium cyanoborohydride, borane pyridine complex, sodium borohydride, borane dimethylamine complex, borane trimethylamine complex, and sodium triacetoxyborohydride. An adequate reducing agent may be selected depending on the kinds of the physiologically active polypeptide or the immunoglobulin constant region and the reaction solvent.

- [24] The reducing agent is used for conjugation of a physiologically active polypeptide or an immunoglobulin constant region with a non-peptidyl polymer. The reaction solution may contain the reducing agent in a concentration of 1 - 20 mM for a reaction between the physiologically active polypeptide or the immunoglobulin constant region and the non-peptidyl polymer, and in a concentration of 1 - 100 mM for the coupling reaction. More preferably, the reducing agent may be used in a concentration of 1 - 20 mM for the conjugation between the physiologically active polypeptide or the immunoglobulin constant region, and the non-peptidyl polymer, and in a concentration of 1 - 40 mM for the coupling reaction.
- [25] The conjugation reaction between a physiologically active polypeptide or the immunoglobulin constant region, and a non-peptidyl polymer (the reaction of step (1)) may be conducted for 1 to 16 hrs and at a temperature of from 0 to 25°C. In addition, the coupling reaction (the reaction of step (2)) may be conducted for 1 to 48 hrs.
- [26] Preferably, the reaction of step (1) may be carried out for 1 to 16 hrs at 0 to 25°C in the presence of a reducing agent in a concentration of 1 to 20 mM while the reaction of step (2) may be carried out for 1 to 48 hrs in the presence of a reducing agent in a concentration of 1 to 40 mM.
- [27] In one preferred embodiment, sodium cyanoborohydride was used as a reducing agent at a variety of conditions, in order to increase the production yield of a complex in which insulin, PEG linker having two or more aldehydes as functional groups, and immunoglobulin constant region are connected together. It has been found that the yield of the coupling reaction is enhanced when the first reaction, which is performed to connect the physiologically active polypeptide or the immunoglobulin constant region and the non-peptidyl polymer, is conducted for a short time at a low temperature in the presence of a low concentration of the reducing agent (Table 1).
- [28] In another preferred embodiment, the reaction was performed with various concentrations of the reducing agent borane pyridine complex, and higher yields of the second

reaction, the coupling reaction, were detected after lower concentrations of the reducing agent were used in the first reaction between a physiologically active polypeptide or an immunoglobulin constant region and a non-peptidyl polymer. The case where Sodium cyanoborohydride was used as a reducing agent shows higher yields compared to the case where borane pyridine complex was used (Table 2).

[29] Moreover, it has been confirmed that the yield of the second, coupling reaction was increased as the concentration of a reducing agent was increased.

[30] In one preferred embodiment, the coupling reaction was carried out at various concentrations of the reducing agent sodium cyanoborohydride, and improved yields of the coupling reaction were obtained in the presence of a high concentration of the reducing agent. However, a very high concentration of the reducing agent caused the immunoglobulin constant region to undergo aberration. In order to avoid this, the coupling reaction was carried out for 13 hrs in the presence of 20 mM sodium cyanoborohydride and it was observed that its yield was maintained at a high level and the aberration of the immunoglobulin constant region was consequently minimized (Tables 3 and 4).

[31] As used herein, the term "physiologically active polypeptide" refers a polypeptide having a certain physiological function in vivo as a general concept. It has a polypeptidyl structure in common and shows various biological activities. When the body becomes biologically abnormal as a result of a lack or an excess of a material involved in a certain function, the physiologically active polypeptide may regulate the genetic expression or physiological function, thereby correcting the abnormality. A typical example is a protein drug.

[32] Examples of the physiologically active polypeptides that are applied in the present invention include human growth hormone, growth hormone releasing hormones, growth hormone releasing peptides, interferon, interferon receptors, colony-stimulating factors, glucagon-like peptides (GLP-1, etc.), oxyntomodulin, G protein-coupled receptors, interleukins, interleukin receptors, enzymes, interleukin-binding proteins, cytokine-binding proteins, macrophage activating factors, macrophage peptides, B-cell factors, T-cell factors, Protein A, allergy inhibitors, cell necrosis glycoproteins, immunotoxins, lymphotoxins, tumor necrosis factor, tumor suppressors, transforming growth factor, alpha-1 anti-trypsin, albumin, α -lactalbumin, apolipoprotein-E, erythropoietin, glycosylated erythropoietin, angiopoietins, hemoglobin, thrombin, thrombin receptor activating peptides, thrombomodulin, blood factor VII, VIIa, VIII, IX and XIII, plasminogen activators, fibrin-binding peptides, urokinase, streptokinase, hirudin, Protein C, C-reactive protein, renin inhibitor, collagenase inhibitor, superoxide dismutase, leptin, platelet-derived growth factor, epithelial growth factor, epidermal growth factor, angiotensin, angiotensin, bone growth factor, bone stimulating protein,

calcitonin, insulin, atriopeptin, cartilage inducing factor, elcatonin, connective tissue activating factor, tissue factor pathway inhibitor, follicle stimulating hormone, luteinizing hormone, luteinizing hormone releasing hormone, nerve growth factors, parathyroid hormone, relaxin, secretin, somatomedin, insulin-like growth factor, adrenocortical hormone, glucagon, cholecystokinin, pancreatic polypeptide, gastrin releasing peptide, corticotropin releasing factor, thyroid stimulating hormone, autotaxin, lactoferrin, myostatin, cell surface antigens, virus-derived vaccine antigens, monoclonal antibodies, polyclonal antibodies, and antibody fragments.

[33] Insulin used in the embodiment of the present invention is a kind of physiologically active peptides secreted from the pancreas when blood glucose level becomes high, which functions to control blood glucose levels by causing the liver, skeletal muscles, and fat tissue to take up glucose from the blood and store it as glycogen, and by suppressing lipolysis, a metabolism for using fat as an energy source. The physiologically active peptides include insulin agonists, precursors, derivatives, fragments, and variants. Native insulin, fast-acting insulin, and long-acting insulin are preferred.

[34] Native insulin is a hormone secreted from the pancreas and plays a critical role in the control of blood glucose levels by promoting the cellular uptake of glucose and inhibiting lipolysis. Insulin having a function of regulating blood glucose levels is produced from a proinsulin precursor without a function of regulating blood glucose levels, through a series of the processes. The amino acid sequence is as follows:

[35] -Alpha Chain:

[36] Gly-Ile-Val-Glu-Gln-Cys-Cys-Thr-Ser-Ile-Cys-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn (SEQ ID NO: 1)

[37] -Beta Chain:

[38] Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr (SEQ ID NO: 2)

[39]

[40] The term "insulin agonist," as used herein, refers to a substance which can bind to an in vivo insulin receptor and exhibit the same biological activity as that of insulin regardless of the structure of insulin.

[41] The term "insulin derivative," as used herein, refers to a peptide that functions to control blood glucose levels in vivo and has an amino acid sequence which shares at least 80% homology with native insulin. There may be a chemical substitution (e.g., alpha-methylation, alpha-hydroxylation), deletion (e.g., deamination) or modification (e.g., N-methylation, glycosylation, fatty acid) at some amino acid residues.

[42] As used herein, the term "insulin fragment" refers to a peptide having the function of controlling blood glucose levels in vivo, prepared by adding at least one amino acid to or deleting at least one amino acid from the amino or carboxyl terminus of insulin. The

- added amino acid may be a non-naturally occurring amino acid (e.g., D-amino acid).
- [43] As used herein, the term "insulin variant" refers to a peptide having the amino acid sequence of which is different from that of native insulin by one or more amino acid residue but having a function of controlling blood glucose levels in vivo.
- [44] In addition, the methods used respectively for the preparation of insulin agonists, fragments and variants may be employed independently or in combination. For example, the insulin peptide useful in the present invention may include a peptide, having the amino acid sequence of which is different from that of native insulin by one or more amino acid residue, with deamination at the N-terminal residue, but functioning to control blood glucose levels in vivo.
- [45] As used herein, the term "immunoglobulin constant region" refers to an immunoglobulin fragment that is devoid of the variable regions of light and heavy chains, the constant region 1 of the heavy chain (C_{H1}), and the constant region of the light chain (C_L), that is, an Fc region comprised of the constant regions 2 and 3 of the heavy chain (C_{H2} and C_{H3}) (or inclusive of the constant region of the heavy chain (C_{H4})). Optionally, the immunoglobulin Fc region may further comprise a hinge region. Also, the immunoglobulin constant region of the present invention may be an extended immunoglobulin Fc region which comprises a part of or the entirety of the constant region 1 of the heavy chain (C_{H1}) and/or the constant region of the light chain (C_L) except only for the variable regions of heavy and light chains of the immunoglobulin so long as it shows effects substantially identical or superior to those of the native immunoglobulin constant region. Further, the immunoglobulin constant region of the present invention may be lack of a significant part of the amino acid sequence which corresponds to C_{H2} and/or C_{H3} . Consequently, the immunoglobulin constant region of the present invention may comprise (1) C_{H1} domain, C_{H2} domain, C_{H3} domain and C_{H4} domain, (2) C_{H1} domain and C_{H2} domain, (3) C_{H1} domain and C_{H3} domain, (4) C_{H2} domain and C_{H3} domain, (5) a combination of one or more constant domains and an immunoglobulin hinge region (or a partial hinge region), or (6) a dimer of each constant domain of the heavy chain and the constant region of the light chain.
- [46] An immunoglobulin constant region including Fc region is a biodegradable polypeptide which can be metabolized in vivo, so that it can safely be used as a drug carrier. In addition, an immunoglobulin Fc region is more advantageous in terms of production, purification and production yield of a complex than an entire immunoglobulin molecule owing to its relatively lower molecular weight. Further, since it is devoid of Fab, which exhibits high non-homogeneity due to the difference in amino acid sequence from one antibody to another, the immunoglobulin Fc alone provides the complex with significantly enhanced homogeneity, and reduces the possibility of inducing blood antigenicity.

- [47] The immunoglobulin constant region may originate from humans or animals, such as cows, goats, pigs, mice, rabbits, hamsters, rats, guinea pigs, etc., and may be preferably of human origin. In addition, the immunoglobulin constant region may be selected from Fc fragments derived from IgG, IgA, IgD, IgE, IgM, or combinations or hybrids thereof. Preferably, the constant region is derived from IgG or IgM, which are the most abundant ones in blood, and most preferably from IgG, which is known to improve the serum half life of ligand-binding proteins.
- [48] As used herein, the term "combination" means that polypeptides encoding single-chain immunoglobulin constant regions (preferably Fc regions) of the same origin are linked to a single-chain polypeptide of a different origin to form a dimer or multimer. That is, a dimer or a multimer may be prepared by combination of two or more fragments selected from the group consisting of fragments of IgG Fc, IgA Fc, IgM Fc, IgD Fc and IgE Fc.
- [49] As used herein, the term "hybrid" means that sequences encoding two or more immunoglobulin Fc fragments of different origins are present in a single-chain of immunoglobulin constant region (preferably, an Fc region). In the present invention, various hybrid forms are possible. For example, the hybrid domain can be composed of one to four domains selected from the group consisting of C_{H1}, C_{H2}, C_{H3} and C_{H4} of IgG Fc, IgM Fc, IgA Fc, IgE Fc and IgD Fc, and may comprise a hinge region.
- [50] IgG is divided into the IgG1, IgG2, IgG3 and IgG4 subclasses, and the present invention may include combinations or hybrids thereof. Preferred are the IgG2 and IgG4 subclasses, and most preferred is the Fc region of IgG4 rarely having effector functions such as Complement Dependent Cytotoxicity (CDC).
- [51] The immunoglobulin constant region may have the glycosylated form to the same extent as, or in a higher or lesser extent than the native form or may be the deglycosylated form. Increased or decreased glycosylation or deglycosylation of the immunoglobulin region may be achieved by typical methods, for example, by using a chemical method, an enzymatic method or a genetic engineering method using microorganisms. Herein, when deglycosylated, the complement (C1q) binding to an immunoglobulin Fc region becomes significantly decreased and antibody-dependent cytotoxicity or complement-dependent cytotoxicity is reduced or removed, thereby not inducing unnecessary immune responses in vivo. In this context, deglycosylated or aglycosylated immunoglobulin Fc regions are more consistent with the purpose of drug carriers. Accordingly, the immunoglobulin Fc region which is the most suitable as the drug carrier of the present invention is a human IgG4-derived aglycosylated Fc region. The human-derived Fc region is more preferable than a non-human derived Fc region, which may act as an antigen in the human body and cause undesirable immune responses such as the production of a new antibody against the antigen.

- [52] Further, not only the immunoglobulin constant region with the native amino acid sequence but also its amino acid sequence mutant may be included within the scope of the immunoglobulin constant region of the present invention. The term "amino acid sequence mutant," as used herein, refers to polypeptides having an amino acid sequence that is different from the wild-type as a result of deletion, insertion, conserved or non-conserved substitution of one or more amino acid residues, or a combination thereof. For instance, amino acid residues at positions 214 to 238, 297 to 299, 318 to 322, or 327 to 331 in IgG Fc, known to be important for linkage, may be used as the sites suitable for modification. Various derivatives, such as those prepared by removing the sites capable of forming disulfide bonds, removing several N-terminal amino acids from native Fc, or adding methionine to the N-terminus of native Fc, may be used in the present invention. In addition, complement fixation sites, e.g., C1q fixation sites, or ADCC sites may be eliminated from the native Fc region to remove the effector function. The techniques of preparing amino acid sequence mutants of the immunoglobulin constant region are disclosed in International Patent Publication Nos. WO 97/34631 and WO 96/32478.
- [53] Amino acid substitutions in a protein or peptide molecule that do not alter the activity of the molecule are well known in the art (H.Neurath, R.L.Hill, *The Proteins*, Academic Press, New York, 1979). The most common substitutions occur between amino acid residues Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Thr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly. Optionally, amino acids may be modified by phosphorylation, sulfation, acrylation, glycosylation, methylation, farnesylation, acetylation, and amidation.
- [54] The above-described immunoglobulin constant region derivatives exhibit the same biological activity as that of the immunoglobulin constant region of the present invention, but have improved structural stability against heat, pH and so forth. These immunoglobulin constant regions may be obtained from native type isolated from humans or animals such as cow, goats, pigs, mice, rabbits, hamsters, rats, guinea pigs, etc., or may be their recombinants or derivatives obtained from transformed animal cells or microorganisms. Native constant regions may be obtained by protease digestion of the entire gamut of immunoglobulins isolated from human or animal samples. Immunoglobulins are cleaved into Fab and Fc by papain and into pF'c and F(ab)₂ by pepsin, followed by size-exclusion chromatography to separate Fc or pF'c therefrom.
- [55] Preferable is a recombinant human immunoglobulin constant region obtained from a microorganism.
- [56]

Advantageous Effects of Invention

[57] As described above, a complex of physiologically active polypeptide-non-peptidyl polymer-immunoglobulin constant region can be produced at high purity and yield as well as at low cost by the method of the present invention. Thus, the method of the present invention is industrially useful. Moreover, it can be used to develop long-acting formulations of physiologically active polypeptides which have improved drug compliance.

[58]

Mode for the Invention

[59] A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as limiting the present invention.

[60]

[61] EXAMPLE 1: PEGylation of Insulin Using Sodium Cyanoborohydride as Reducing Agent and Purification of Mono-PEGylated Insulin

[62]

[63] Insulin powder was dissolved in 10 mM HCl, and PEGylated at the N-terminus of the beta chain with 3.4K propion-ALD2 PEG (PEG with two propionaldehyde groups, IDB, Korea). In this regard, 5 mg/ml insulin was reacted with PEG at a molar ratio of 1:2 at 4°C to room temperature for 2 hrs. The reaction was performed in 50 mM sodium citrate buffer at pH 6.0 in 45% isopropanol in the presence of 2 - 20 mM sodium cyanoborohydride as a reducing agent. The reaction mixture was loaded onto an SP-HP (GE Healthcare) column, followed by eluting with a buffer containing sodium citrate (pH 3.0) and 45% EtOH, and using a concentration gradient of KCl to purify mono-PEGylated insulin.

[64] PEGylation yields of insulin according to conditions of the reducing agent sodium cyanoborohydride during the preparation of a complex comprising the insulin and the immunoglobulin Fc region are summarized in Table 1, below.

[65]

[66] EXAMPLE 2: Changes in Production Yields of a Complex of Mono-PEGylated Insulin-Immunoglobulin Fc Region According to Conditions of Sodium Cyanoborohydride as Reducing Agent Used in PEGylation

[67]

[68] To examine the production yield of the insulin-PEG-immunoglobulin Fc region complex, the mono-PEGylated insulin prepared in Example 1 was reacted with at a molar ratio of 1:1 with an immunoglobulin Fc at 25°C for 13 hrs, with the total protein concentration set to be 20 mg/ml. This coupling reaction was carried out in 100 mM

HEPES buffer containing 22 mM potassium phosphate and 10% ethanol at pH 8.2, in the presence of 20 mM sodium cyanoborohydride as a reducing agent.

[69] The reaction mixture was loaded onto a Source 15Q (GE Healthcare) column, followed by eluting with Tris-HCl (pH 7.5) buffer and using a concentration gradient of NaCl to separate and purify unreacted insulin, unreacted immunoglobulin Fc region, an insulin-PEG-immunoglobulin Fc region complex, and an immunoglobulin Fc region coupled with two or more mono-PEGylated insulin (insulin-PEG) moieties. Production yields of the insulin-PEG-immunoglobulin Fc region complex were determined UV absorbance at 280 nm after purification by chromatography.

[70] In Table 1, yields of the coupling reaction with an immunoglobulin Fc region are summarized according to conditions of sodium cyanoborohydride used as a reducing agent in the PEGylation of insulin.

[71]

[72] Table 1

[Table 1]

Conc. of Sodium cyanoborohydride	RxnTime	Rxn Temp.	PEGylation yield (%)	Coupling yield (%)	Total yield(%)
2 mM	2hr	4°C	23.3	31.7	7.39
4 mM	2hr	4°C	37.6	31	11.66
4 mM	2hr	RT	39.2	28.8	11.29
8 mM	2hr	RT	40.4	27.1	10.95
8 mM	4hr	4°C	40.4	27	10.9
20 mM	2hr	4°C	42.2	26.8	11.3

[73]

[74] **EXAMPLE 3: PEGylation of Insulin Using Borane Pyridine Complex as Reducing Agent and Purification of Mono-PEGylated Insulin**

[75]

[76] Insulin powder was dissolved in 10 mM HCl, and PEGylated at the N-terminus of the beta chain with 3.4K propion-ALD2 PEG (PEG with two propionaldehyde groups, IDB, Korea). In this regard, 5 mg/ml insulin was reacted with PEG at a molar ratio of 1:2 at 4°C for 2 hrs. The reaction was performed in 50 mM sodium citrate buffer at pH 6.0 in 45% isopropanol in the presence of 3 - 20 mM borane pyridine complex as a reducing agent. The reaction mixture was loaded onto an SP-HP (GE Healthcare) column, followed by eluting with a buffer containing sodium citrate (pH 3.0) and 45% EtOH, and using a concentration gradient of KCl to purify mono-PEGylated insulin.

[77] PEGylation yields of insulin according to conditions of the reducing agent borane pyridine complex during the preparation of a complex comprising the insulin and the immunoglobulin Fc region are summarized in Table 2, below.

[78]

[79] **EXAMPLE 4: Changes in Production Yields of a Complex of Mono-PEGylated Insulin-Immunoglobulin Fc Region According to Conditions of Borane Pyridine Complex as Reducing Agent Used in PEGylation**

[80]

[81] To examine the production yield of the insulin-PEG-immunoglobulin Fc region complex, the mono-PEGylated insulin prepared in Example 3 was reacted with at a molar ratio of 1:1 with an immunoglobulin Fc at 25°C for 13 hrs, with the total protein concentration set to be 20 mg/ml. This coupling reaction was carried out in 100 mM HEPES buffer containing 22 mM potassium phosphate and 10% ethanol at pH 8.2, in the presence of 20 mM sodium cyanoborohydride as a reducing agent.

[82] The reaction mixture was loaded onto a Source 15Q (GE Healthcare) column, followed by eluting with Tris-HCl (pH 7.5) buffer and using a concentration gradient of NaCl to separate and purify unreacted insulin, unreacted immunoglobulin Fc region, an insulin-PEG-immunoglobulin Fc region complex, and an immunoglobulin Fc region coupled with two or more mono-PEGylated insulin (insulin-PEG) moieties. Production yields of the insulin-PEG-immunoglobulin Fc region complex were determined UV absorbance at 280 nm after purification by chromatography.

[83] In Table 2, yields of the coupling reaction with an immunoglobulin Fc region are summarized according to conditions of borane pyridine complex used as a reducing agent in the PEGylation of insulin.

[84]

[85] Table 2

[Table 2]

Conc. of Borane pyridine complex	PEGylation yield (%)	Coupling yield (%)	Total yield (%)
3 mM	25.4	35.1	8.92
10 mM	47.6	34.8	16.6
20 mM	50.8	34.2	17.4

[86]

[87] **EXAMPLE 5: Yields of Coupling Reaction and Formation of Immunoglobulin Fc Aberrant According to Concentration of Sodium Cyanoborohydride and Reaction Time**

[88]

[89] To examine the formation of immunoglobulin Fc aberrant according to the concentrations of the reducing agent, and reaction times in the coupling reaction, the mono-PEGylated insulin was reacted at a molar ratio of 1:1 with an immunoglobulin Fc at 25°C for 13 - 43 hrs, with the total protein concentration set to be 20 mg/ml. This coupling reaction was carried out in 100 mM HEPES buffer containing 22 mM potassium phosphate and 10% ethanol, pH 8.2, in the presence of 5 - 40 mM sodium cyanoborohydride.

[90] The reaction mixture was loaded onto a Source 15Q (GE Healthcare) column, followed by eluting with Tris-HCl (pH 7.5) buffer and using a concentration gradient of NaCl to separate and purify unreacted insulin, unreacted immunoglobulin Fc region, an insulin-PEG-immunoglobulin Fc region complex, and an immunoglobulin Fc region coupled with two or more mono-PEGylated insulin (insulin-PEG) moieties. Production yields of the insulin-PEG-immunoglobulin Fc region complex were determined UV absorbance at 280 nm after purification by chromatography.

[91] In Table 3, yields of the coupling reaction to prepare a complex comprising the insulin and the immunoglobulin Fc region are summarized according to concentrations of sodium cyanoborohydride used as a reducing agent, and reaction times in the coupling reaction.

[92]

[93] Table 3

[Table 3]

Rxn Time	5 mM SCB	20 mM SCB	40 mM SCB
13 hrs	35.2%	37.5%	37.5%
18 hrs	36.1%	37.7%	37.7%
37 hrs	36.7%	37.3%	37.5%
43 hrs	36.8%	37.2%	36.8%

[94]

[95] The formation of immunoglobulin Fc aberrants according to the concentrations of the reducing agent in the coupling reaction was monitored by LC on a Propac SAX-10 (DIONEX) column eluting with Tris-HCl (pH 8.0) buffer and using a concentration gradient of NaCl.

[96] In Table 4, production yields of immunoglobulin Fc aberrants are given according to concentrations of sodium cyanoborohydride and reaction times in the coupling reaction to prepare a complex comprising the insulin and the immunoglobulin Fc region.

[97]

[98] Table 4

[Table 4]

Rxn Time	5 mM SCB	20 mM SCB	40 mM SCB
13 hrs	4.6%	7.0%	7.8%
18 hrs	6.2%	9.0%	9.8%
37 hrs	11.7%	14.5%	15.3%
43 hrs	12.7%	15.5%	16.8%

[99]

[100] **EXAMPLE 6: PEGylation of Immunoglobulin Fc Using Sodium Cyanoborohydride as Reducing Agent and Purification of Mono-PEGylated Immunoglobulin Fc**

[101]

[102] The N-terminus of immunoglobulin Fc was PEGylated with 5K propion-ALD2 PEG (PEG with three propionaldehyde groups, NOF, Japan). In this regard, 10 mg/ml immunoglobulin Fc was reacted with PEG at a molar ratio of 1:2 at 4°C to room temperature for 4.5 hrs. The reaction was performed in 100 mM potassium phosphate buffer at pH 6.0 in the presence of 20 mM sodium cyanoborohydride as a reducing agent. The reaction mixture was loaded onto a Source 15Q column, followed by eluting with Tris-HCl (pH 7.5) buffer and using a concentration gradient of NaCl to purify mono-PEGylated insulin.

[103]

[104] **EXAMPLE 7: Preparation of a Complex of Mono-PEGylated Immunoglobulin Fc Region-Insulin Using Sodium Cyanoborohydride as Reducing Agent**

[105]

[106] To prepare an insulin-PEG-immunoglobulin Fc region complex, the mono-PEGylated immunoglobulin Fc prepared in Example 6 was reacted with at a molar ratio of 1:4 with an insulin at 4°C for 13 hrs, with the total protein concentration set to be 20 mg/ml. This coupling reaction was carried out in 100 mM potassium phosphate buffer at pH 6.0 in the presence of 20 mM sodium cyanoborohydride as a reducing agent.

[107] The reaction mixture was loaded onto a Source 15Q (GE Healthcare) column for the primary purification. And the secondary purification was additionally performed with a Source 15ISO (GE Healthcare) column to obtain an insulin-PEG-immunoglobulin Fc region complex.

[108]

Claims

- [Claim 1] A method for preparing a complex of physiologically active polypeptide-non-peptidyl polymer-immunoglobulin constant region, comprising:
- (1) reacting a non-peptidyl polymer having two or more aldehydes as functional groups with one of a physiologically active polypeptide or an immunoglobulin constant region in the presence of a reducing agent at a concentration of 1 - 20 mM; and
 - (2) reacting the reaction mixture of step (1) with the other of a physiologically active polypeptide or immunoglobulin constant region in the presence of a reducing agent at a concentration of 1 - 100 mM.
- [Claim 2] The method of claim 1, further comprising separating a conjugate of a physiologically active polypeptide-non-peptidyl polymer or a conjugate of an immunoglobulin constant region-non-peptidyl polymer from the reaction mixture after step (1).
- [Claim 3] The method of claim 1, wherein the reducing agent functions to reduce a reversible imine double bond produced from bonding between the aldehyde group of the non-peptidyl polymer and an amine group of the physiologically active polypeptide or the immunoglobulin constant region to form a covalent bond.
- [Claim 4] The method of claim 1, wherein the reducing agent is selected from the group consisting of sodium cyanoborohydride, borane pyridine complex, sodium borohydride, borane dimethylamine complex, borane trimethylamine complex, and sodium triacetoxyborohydride.
- [Claim 5] The method of claim 1, wherein the reducing agent is used in a concentration of 1 to 40 mM in step (2).
- [Claim 6] The method of claim 1, wherein the reaction in step (1) is carried out for 1 to 16 hrs.
- [Claim 7] The method of claim 1, wherein the reaction in step (2) is carried out for 1 to 48 hrs.
- [Claim 8] The method of claim 1, wherein the reaction in step (1) is carried out at a temperature of 0 to 25°C.
- [Claim 9] The method of claim 1, wherein the reaction in step (1) is carried out for 1 to 16 hrs at 0 to 25°C in the presence of the reducing agent in a concentration of 1 to 20 mM, and the reaction in step (2) is carried out for 1 to 48 hrs in the presence of the reducing agent in a concentration of 1 to 40 mM.

- [Claim 10] The method of claim 1, wherein the non-peptidyl polymer is covalently bonded to each of the physiologically active polypeptide and the immunoglobulin constant region through the two or more aldehyde functional groups thereof.
- [Claim 11] The method of claim 1, wherein the functional groups of the non-peptidyl polymer are bonded to each of an amine group of the physiologically active polypeptide and the immunoglobulin constant region, wherein the amine group is present at an N-terminus or on a side chain of Lys residue.
- [Claim 12] The method of claim 1, wherein the non-peptidyl polymer is selected from the group consisting of polyethylene glycols, polypropylene glycols, copolymers of ethylene glycol and propylene glycol, polyoxyethylated polyols, polyvinyl alcohols, polysaccharides, dextrans, polyvinyl ethyl ethers, polylactic acid (PLA), polylactic-glycolic acid (PLGA), lipid polymers, chitins, hyaluronic acid, and the combination thereof.
- [Claim 13] The method of claim 1, wherein the non-peptidyl polymer is polyethylene glycols.
- [Claim 14] The method of claim 1, wherein molecular weight of the non-peptidyl polymer ranges from 1 to 100 kDa.
- [Claim 15] The method of claim 1, wherein the immunoglobulin constant region is aglycosylated.
- [Claim 16] The method of claim 1, wherein the immunoglobulin constant region consists of one to four domains selected from the group consisting of C_{H1}, C_{H2}, C_{H3} and C_{H4} domains.
- [Claim 17] The method of claim 1, wherein the immunoglobulin constant region further comprises a hinge region.
- [Claim 18] The method of claim 1, wherein the immunoglobulin constant region is selected from the group consisting of constant regions derived from IgG, IgA, IgD, IgE, IgM, or combinations or hybrids thereof.
- [Claim 19] The method of claim 1, wherein the immunoglobulin constant region is selected from the group consisting of constant regions of IgG1, IgG2, IgG3, IgG4, a combination thereof, and a hybrid thereof.
- [Claim 20] The method of claim 1, wherein the immunoglobulin constant region is an IgG4 Fc region.
- [Claim 21] The method of claim 20, wherein the immunoglobulin constant region is an aglycosylated human IgG4 Fc region.
- [Claim 22] The method of claim 1, wherein the physiologically active polypeptide

is selected from the group consisting of human growth hormone, growth hormone releasing hormones, growth hormone releasing peptides, interferon, interferon receptors, colony-stimulating factors, glucagon-like peptides (GLP-1, etc.), oxyntomodulin, G protein-coupled receptors, interleukins, interleukin receptors, enzymes, interleukin-binding proteins, cytokine-binding proteins, macrophage activating factors, macrophage peptides, B-cell factors, T-cell factors, Protein A, allergy inhibitors, cell necrosis glycoproteins, immunotoxins, lymphotoxins, tumor necrosis factor, tumor suppressors, transforming growth factor, alpha-1 anti-trypsin, albumin, α -lactalbumin, apolipoprotein-E, erythropoietin, glycosylated erythropoietin, angiopoietins, hemoglobin, thrombin, thrombin receptor activating peptides, thrombomodulin, blood factor VII, VIIa, VIII, IX and XIII, plasminogen activators, fibrin-binding peptides, urokinase, streptokinase, hirudin, Protein C, C-reactive protein, renin inhibitor, collagenase inhibitor, superoxide dismutase, leptin, platelet-derived growth factor, epithelial growth factor, epidermal growth factor, angiostatin, angiotensin, bone growth factor, bone stimulating protein, calcitonin, insulin, atriopeptin, cartilage inducing factor, elcatonin, connective tissue activating factor, tissue factor pathway inhibitor, follicle stimulating hormone, luteinizing hormone, luteinizing hormone releasing hormone, nerve growth factors, parathyroid hormone, relaxin, secretin, somatomedin, insulin-like growth factor, adrenocortical hormone, glucagon, cholecystokinin, pancreatic polypeptide, gastrin releasing peptide, corticotropin releasing factor, thyroid stimulating hormone, autotaxin, lactoferrin, myostatin, cell surface antigens, virus-derived vaccine antigens, monoclonal antibodies, polyclonal antibodies, and antibody fragments.

[Claim 23]

The method of claim 1, wherein the physiologically active polypeptide is insulin.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2013/001885**A. CLASSIFICATION OF SUBJECT MATTER****C07K 19/00(2006.01)i, C07K 1/10(2006.01)i, A61K 47/48(2006.01)i**

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K 19/00; A61K 39/395; A61K 31/74; C07K 16/46; A61K 38/16; A61K 38/26; A61K 31/4164

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & Keywords: polypeptide-non-peptidyl polymer-immunoglobulin constant region, PEG, insulin, physiologically active polypeptide complex**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010-011096 A2 (HANMI PHARMACEUTICAL CO., LTD.) 28 January 2010 See claims 11 and 15; paragraphs [0030], [0045], [0063], [0068], [0088] and [0111].	1-23
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A	WO 2012-011752 A2 (HANMI HOLDINGS CO., LTD.) 26 January 2012 See claims 1, 6-9, 13 and 15.	1-23



Further documents are listed in the continuation of Box C.



See patent family annex.

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"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

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2013/001885

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of:

a. a sequence listing filed or furnished

- ☐ on paper
☒ in electronic form

b. time of filing or furnishing

- ☒ contained in the international application as filed
☐ filed together with the international application in electronic form
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2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2013/001885

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(54) 发明名称

制备生理活性多肽复合物的改进方法

(57) 摘要

本发明公开的是一种制备复合物的方法,该复合物中的生理活性多肽通过非肽基连接体共价结合至免疫球蛋白恒定区。该方法的特征在于还原剂的使用,由此可以克服传统的低产率和多肽修饰的问题。可以以高纯度和高产率以及低成本生产生理活性多肽-非肽基聚合物-免疫球蛋白恒定区复合物。因此,该方法在工业上是有用的。而且,通过表现出长效特征,该生理活性多肽-非肽基聚合物-免疫球蛋白恒定区复合物可以有效地用于开发具有改善的药物顺应性的生理活性多肽的长效制剂。

1. 制备生理活性多肽-非肽基聚合物-免疫球蛋白恒定区复合物的方法,其包括:
 - (1) 使具有两个或更多个醛作为官能团的非肽基聚合物与生理活性多肽或免疫球蛋白恒定区的其中之一在浓度为 1-20mM 的还原剂的存在下反应;和
 - (2) 使步骤 (1) 的反应混合物与生理活性多肽或免疫球蛋白恒定区中的另一个在浓度为 1-100mM 的还原剂的存在下反应。
2. 权利要求 1 所述的方法,进一步包括:在步骤 (1) 之后,从所述反应混合物中分离出生理活性多肽-非肽基聚合物的结合物或免疫球蛋白恒定区-非肽基聚合物的结合物。
3. 权利要求 1 所述的方法,其中所述还原剂的作用是还原在所述非肽基聚合物的所述醛基与所述生理活性多肽或所述免疫球蛋白恒定区的胺基之间结合所产生的可逆性亚胺双键以形成共价键。
4. 权利要求 1 所述的方法,其中所述还原剂选自氰基硼氢化钠、硼烷吡啶复合物、硼氢化钠、硼烷二甲基胺复合物、硼烷三甲基胺复合物和三乙酰氧基硼氢化钠。
5. 权利要求 1 所述的方法,其中在步骤 (2) 中,所述还原剂的使用浓度为 1 至 40mM。
6. 权利要求 1 所述的方法,其中步骤 (1) 中的所述反应进行 1 至 16 小时。
7. 权利要求 1 所述的方法,其中步骤 (2) 中的所述反应进行 1 至 48 小时。
8. 权利要求 1 所述的方法,其中步骤 (1) 中的所述反应在 0 至 25℃ 的温度下进行。
9. 权利要求 1 所述的方法,其中步骤 (1) 中的所述反应在 0 至 25℃ 下,在浓度为 1 至 20mM 的所述还原剂的存在下进行 1 至 16 小时,并且步骤 (2) 中的所述反应在浓度为 1 至 40mM 的所述还原剂的存在下进行 1 至 48 小时。
10. 权利要求 1 所述的方法,其中所述非肽基聚合物通过其两个或更多个醛官能团共价连接至所述生理活性多肽和所述免疫球蛋白恒定区的每一个。
11. 权利要求 1 所述的方法,其中所述非肽基聚合物的所述官能团连接至所述生理活性多肽和所述免疫球蛋白恒定区的各自的胺基,其中所述胺基存在于 N-末端或 Lys 残基的侧链上。
12. 权利要求 1 所述的方法,其中所述非肽基聚合物选自聚乙二醇、聚丙二醇、乙二醇和丙二醇的共聚物、聚氧乙基化多元醇、聚乙烯醇、多糖、葡聚糖、聚乙基乙基醚、聚乳酸(PLA)、聚乳酸-羟基乙酸(PLGA)、脂质聚合物、壳多糖、玻尿酸、和其组合。
13. 权利要求 1 所述的方法,其中所述非肽基聚合物是聚乙二醇。
14. 权利要求 1 所述的方法,其中所述非肽基聚合物的分子量范围是从 1 至 100kDa。
15. 权利要求 1 所述的方法,其中所述免疫球蛋白恒定区是无糖基化的。
16. 权利要求 1 所述的方法,其中所述免疫球蛋白恒定区由一个至四个结构域组成,所述一个至四个结构域选自 C_{H1}、C_{H2}、C_{H3} 和 C_{H4} 结构域。
17. 权利要求 1 所述的方法,其中所述免疫球蛋白恒定区进一步包括铰链区。
18. 权利要求 1 所述的方法,其中所述免疫球蛋白恒定区选自衍生自 IgG、IgA、IgD、IgE、IgM 的恒定区、或其组合或杂化物。
19. 权利要求 1 所述的方法,其中所述免疫球蛋白恒定区选自 IgG1、IgG2、IgG3、IgG4 的恒定区、其组合、和其杂化物。
20. 权利要求 1 所述的方法,其中所述免疫球蛋白恒定区是 IgG4 Fc 区。
21. 权利要求 20 所述的方法,其中所述免疫球蛋白恒定区是无糖基化的人 IgG4 Fc 区。

22. 权利要求 1 所述的方法,其中所述生理活性多肽选自人生长激素、生长激素释放激素、生长激素释放肽、干扰素、干扰素受体、集落刺激因子、类胰高血糖素肽 (GLP-1,等)、胃酸调节素、G 蛋白质 - 偶联受体、白细胞介素、白细胞介素受体、酶、白细胞介素 - 结合蛋白、细胞因子 - 结合蛋白、巨噬细胞活化因子、巨噬细胞肽、B- 细胞因子、T- 细胞因子、蛋白质 A、变态反应抑制剂、细胞坏死糖蛋白、免疫毒素、淋巴毒素、肿瘤坏死因子、肿瘤抑制剂、转化生长因子、 α -1 抗胰蛋白酶、白蛋白、 α - 乳清蛋白、载脂蛋白 -E、促红细胞生成素、糖基化促红细胞生成素、血管生成素、血红素、凝血酶、凝血酶受体活化肽、血栓调节素、血液因子 VII、VIIa、VIII、IX 和 XIII、纤溶酶原激活剂、纤维蛋白 - 结合肽、尿激酶、链球菌激酶、水蛭素、蛋白质 C、C- 反应蛋白、肾素抑制剂、胶原酶抑制剂、超氧化歧化酶、瘦蛋白、血小板 - 衍生长因子、上皮细胞生长因子、表皮细胞生长因子、血管抑制素、血管收缩素、骨生长因子、骨刺激蛋白、降血钙素、胰岛素、心钠素、软骨诱导因子、依降钙素、结缔组织活化因子、组织因子途径抑制剂、卵泡刺激激素、黄体生成素、黄体生成素释放激素、神经生长因子、甲状旁腺激素、松弛肽、分泌素、生长调节素、类胰岛素生长因子、肾上腺皮质激素、胰高血糖素、胆囊收缩素、胰腺多肽、胃泌素释放肽、促肾上腺皮质激素释放因子、甲状腺刺激激素、自毒素、乳铁蛋白、肌肉抑制素、细胞表面抗原、病毒 - 衍生疫苗抗原、单克隆抗体、多克隆抗体和抗体片段。

23. 权利要求 1 所述的方法,其中所述生理活性多肽是胰岛素。

制备生理活性多肽复合物的改进方法

技术领域

[0001] 本发明涉及制备复合物的方法,该复合物中的生理活性多肽通过含两个或更多个醛作为官能团的非肽基聚合物连接体共价结合至免疫球蛋白恒定区。更具体地,本发明涉及有效制备生理活性多肽复合物的方法,其特征在于通过在制备时调整还原剂的使用来改进低产率和多肽试剂变形的问题。

背景技术

[0002] 总体而言,生理活性多肽由于它们的低稳定性而容易变性,并且在血液中容易经历蛋白水解降解和随后的肾或肝脏清除。因此,含生理活性多肽作为药物成分的蛋白质药物需要频繁地对患者给药,以维持适当的血清水平和效价。然而,此类蛋白质药物的频繁给药(其大多数是以注射的形式)引起患者疼痛而且治疗成本高。为了解决这些问题,已经投入许多努力来改进蛋白质药物的血清稳定性,和使血液中的药物长期维持在高水平以使药物的药效达到最大。作为长效制剂使用的需求,蛋白质药物必须配制成具有高稳定性并且维持足够高水平的效价而不引发患者的免疫应答。

[0003] 传统的稳定蛋白质和防止酶降解及肾脏清除的方法是用具有高溶解度的聚合物(例如,聚乙二醇(在下文中称为“PEG”)对蛋白质药物的表面进行化学修饰。通过结合至目标蛋白的特定区域或各种区域,PEG使得蛋白质的溶解度更高,从而稳定蛋白质并防止水解而不引起严重的副作用(Sada等人,J. 发酵生物工程(J.Fermentation Bioengineering)71:137-139)。

[0004] 例如,WO 2006/076471 公开了通过将B-型钠尿肽(BNP)与PEGs结合而使其长效的方法,该PEGs结合至钠尿肽受体A(NPR-A)以引发cGMP的合成,从而降低动脉血压。其因而用于充血性心力衰竭的治疗。美国专利号6,924,264描述了一种通过使其赖氨酸残基PEG化来改进艾塞那肽(exendin-4)在体内的持续时间的方法。然而,在此方法中,尽管其有能力通过增加PEG的分子量来增加肽药物的循环时间,但PEG化仍有问题,例如,大大降低了肽药物的效价和降低了产率,这是因为随着PEG的分子量增加,肽的反应性下降。

[0005] WO 02/46227公开了这样的融合蛋白,其中GLP-1和艾塞那肽(exendin-4)或其类似物与人血清白蛋白或通过遗传重组制备的免疫球蛋白片段(Fc)结合。美国专利号6,756,480涉及这样的融合蛋白,其中甲状旁腺激素(PTH)或其类似物连接至Fc。虽然这些方法可被评估为PEG化问题——例如低产率和非特异性——的解决方案,但是已经显示其增加目标肽的体内半衰期的效果不显著,与预期相反,而且在某些情况下,融合蛋白具有低效价。虽然多种肽基连接体被利用以使增加血清半衰期的效果达到最大,但它们有可能引起免疫应答。此外,具有二硫键的肽(例如,BNP)在实际应用中有困难,因为其很容易引起错误折叠。此外,不可能通过遗传重组产生具有非天然氨基酸残基的肽基连接体。

[0006] 胰岛素是由人胰腺 β -细胞分泌的肽,主要是调节体内血糖水平。当胰岛素分泌不正常或分泌的胰岛素无法在体内适当地发挥作用时,血糖水平不能被控制并且升高,导致糖尿病。后一种情况称为2型糖尿病。然而,在胰岛素不能从胰腺分泌并且引起血糖水

平升高的情况下,导致 1 型糖尿病。2 型糖尿病患者用口服降血糖剂治疗,其中一些患者用胰岛素治疗。同时,对于 1 型糖尿病患者基本上需要注射胰岛素。

[0007] 通常,通过在每餐之前或之后每天注射三次来施用胰岛素进行胰岛素疗法。然而,每天三次连续胰岛素给药对于患者是痛苦的而且不方便。已经做出很多尝试来解决这些问题。旨在提高蛋白质药物在生物膜中的通透性的一种策略是通过口或鼻吸入来递送药物。然而,与注射相比,通过口或鼻吸入给药的递送效率明显地低,并且难以将肽药物维持在体内活性所需的水平。

[0008] 作为可供选择的策略,可以将过量药物皮下注射并以延迟的方式吸收至体内,以维持恒定的血液水平,即使每天注射一次。一些药物(例如,兰德仕(Lantus)、赛诺菲安万特(Sanofi-aventis))已被批准用于商业用途并且目前已对患者给药。另外,已经针对用脂肪酸修饰胰岛素进行了研究以使胰岛素结合物的结合更强并通过在注射位置或血液中与白蛋白组合来延长持续时间。一些药物(例如,诺和平(Levemir)、诺和诺德(NovoNordisk))已被批准用于商业用途。然而,这些药物在注射位置引起疼痛并且必须每天注射一次,这对患者仍是巨大的负担。

[0009] 为了克服现有技术中遇到的问题,本发明者已制备了含生理活性多肽和免疫球蛋白恒定区(其通过使用非肽基聚合物作为连接体连接)的复合物,作为同时增加生理活性多肽(如,胰岛素)的血浆半衰期和体内持续时间的策略。然而,还需要以高产率和高纯度制备复合物的方法,因为该复合物的成分非常昂贵。出于此考虑,本发明者已经开发了以降低的成本、高产率和高纯度制备生理活性多肽复合物的方法,该方法在复合物生产的反应期间,在反应溶液中使用最佳浓度的合适种类的还原剂,从而完成本发明。

[0010] 发明的公开内容

[0011] 技术问题

[0012] 本发明的目的是提供制备复合物的有效方法,其中生理活性多肽通过含两个或更多个醛作为官能团的非肽基聚合物连接体共价结合至免疫球蛋白恒定区,其特征在于在反应期间,通过在反应溶液中使用最佳浓度的合适种类的还原剂来改进低产率和多肽试剂变形的问题。

[0013] 问题解决方案

[0014] 一方面,为了实现上述目的,本发明提供制备生理活性多肽-非肽基聚合物-免疫球蛋白恒定区的复合物的方法,其包括(1)在浓度为 1-20mM 的还原剂的存在下,使具有两个或更多个醛作为官能团的非肽基聚合物与生理活性多肽或免疫球蛋白恒定区的其中之一反应;和(2)使(1)的反应混合物与生理活性多肽或免疫球蛋白恒定区中的另一个在浓度为 1-100mM 的还原剂的存在下反应。

[0015] 该反应混合物可包括非肽基聚合物与生理活性多肽的结合物或非肽基聚合物与免疫球蛋白恒定区的结合物,和/或保持未反应的反应物。因此,本发明的方法可进一步包括:在步骤(1)之后,从反应混合物中分离生理活性多肽-非肽基聚合物的结合物或免疫球蛋白-非肽基聚合物的结合物。

[0016] 本文所用的术语“非肽基聚合物”是指由至少两个重复单元组成的生物相容性聚合物,该重复单元通过除肽键外的任意共价键保持在一起。用于本发明的非肽基聚合物的例子包括:聚乙二醇、聚丙二醇、乙二醇与丙二醇的共聚物、聚氧乙基化多元醇、聚乙烯基

醇、多糖、葡聚糖、聚乙烯基乙醚、生物可降解聚合物（例如，聚乳酸（PLA）和聚乳酸-羟基乙酸（PLGA））、脂质聚合物、壳多糖、玻尿酸及其组合，优选聚乙二醇（PEG）。本领域中众所周知的其衍生物和容易使用本领域已知的方法制备的衍生物均在本发明的范围内。

[0017] 如上所述，非肽基聚合物可具有两个或更多个醛作为官能团。因此，上面所列出的非肽基聚合物本身可以是双-或-多功能醛形式或优选地在其两个或更多个醇基上包含具有醛基的取代基。具有醛基的取代基可以是烷基醛（例如，丙醛或丁醛）。在一个优选实施例中，非肽基聚合物可以是在其每个末端上具有丙醛取代基的 PEG。

[0018] 通过框内融合技术构建的融合蛋白质中所使用的传统肽基连接体的缺点是肽基连接体容易在体内被蛋白酶裂解，因而不能通过载体确保血清半衰期的延长，这与预期相反。然而，在本发明中，使用对蛋白酶抵抗的聚合物，因此肽的血浆半衰期可维持在与载体相似的水平。因此，只要对体内蛋白酶抵抗，任何非肽基聚合物都可用于本发明，而没有限制。非肽基聚合物的分子量范围从 1 至 100kDa，优选于 1 至 20kDa。此外，连接至生理活性多肽的非肽基聚合物可以是单个的聚合物或不同聚合物的组合。而且，用于本发明的非肽基聚合物可以在其两个或三个末端具有可与生理活性多肽和免疫球蛋白恒定区偶联的官能团。优选地，该官能团可以为醛。

[0019] 与 PEG 结合（其通常被用于制备蛋白质药物的长效制剂）增加蛋白质的稳定性，而较大分子量的 PEG 显示与蛋白质的反应性较低，因而降低产率。由于产率与生产成本和工业适用性紧密相关，所以增加产率是非常重要的。用醛作为官能团的 PEG 可偶联至胺基，其存在于生理活性多肽或免疫球蛋白恒定区的 N-末端或 Lys 残基侧链上。在这方面，PEG 化的产率可根据各种因素而变化，这些因素包括 PEG 与蛋白质的摩尔比、反应溶液的浓度、反应时间、pH、温度等。Chem. Biol. Drug Des. 2007 ;69 ;132-138 描述了通过调整各种因素（包括摩尔比、反应时间、pH 等）用 5K 醛 mPEG 进行胰岛素 PEG 化，产率超过 90%。US 2009/0252703A1 中报道了将有机溶剂添加至反应溶液增加肽 PEG 化的产率。WO 2010/089756A2 公开了通过将 r-metHuG-CSF 与 PEG 在碳水化合物的存在下反应以改进 PEG 化产率。

[0020] 然而，当使用含具有两个或更多个官能团的 PEG 的非肽基聚合物作为两个不同多肽之间的连接体时，反应中需要两个或更多个步骤，从而降低总产率。具体地，与第一反应的步骤（其中生理活性多肽或免疫球蛋白恒定区与具有两个或更多个官能团的非肽基聚合物反应）相比，观察到第二反应的步骤（其中与具有两个或更多个官能团的非肽基聚合物结合的生理活性多肽或免疫球蛋白恒定区分别与免疫球蛋白恒定区或生理活性多肽反应，下文中称为“偶联反应”）以明显较低的产率进行。

[0021] 在本发明中，已证明第一反应中的还原剂浓度与第二偶合反应的产率有关。第一反应中的还原剂浓度越低、反应时间越短和反应温度越低时，观察到该偶联反应的产率越高。

[0022] 本文所使用的术语“还原剂”是指这样的化合物，其功能是还原从非肽基聚合物的醛基与多肽（生理活性多肽、免疫球蛋白恒定区）的胺基之间的反应中所形成的可逆亚胺双键，从而形成共价键，并且意图包括所有本领域中已知的还原剂。为了本发明的目的，可将还原剂添加至反应溶液中，其中非肽基聚合物与生理活性多肽或免疫球蛋白恒定区形成共价键。只要是本领域常用的，任何还原剂都可用于本发明。还原剂的例子可包括，但不限

于：氰基硼氢化钠、硼烷吡啶复合物、硼氢化钠、硼烷二甲基胺复合物、硼烷三甲基胺复合物和三乙酰氧基硼氢化钠。可依据生理活性多肽或免疫球蛋白恒定区和反应溶剂的种类选择适当的还原剂。

[0023] 还原剂用于生理活性多肽或免疫球蛋白恒定区与非肽基聚合物的结合。反应溶液可包括用于生理活性多肽或免疫球蛋白恒定区与非肽基聚合物之间的反应的浓度在 1-20mM 的还原剂,和用于偶联反应的浓度在 1-100mM 的还原剂。更优选地,还原剂可以 1-20mM 的浓度用于生理活性多肽或免疫球蛋白恒定区与非肽基聚合物之间的结合反应和以 1-40mM 的浓度用于偶联反应。

[0024] 生理活性多肽或免疫球蛋白恒定区与非肽基聚合物之间的结合反应(步骤(1)的反应)可在 0 至 25℃ 温度下进行 1 至 16 小时。此外,偶联反应(步骤(2)的反应)可进行 1 至 48 小时。

[0025] 优选地,步骤(1)的反应可在 0 至 25℃ 温度下,在浓度为 1 至 20mM 的还原剂的存在下进行 1 至 16 小时,而步骤(2)的反应可在浓度为 1 至 40mM 的还原剂的存在下进行 1 至 48 小时。

[0026] 在一个优选的实施方式中,在各种不同的条件下使用氰基硼氢化钠作为还原剂,以增加复合物的产率,在复合物中,胰岛素、具有两个或更多个醛作为官能团的 PEG 连接体和免疫球蛋白恒定区被连接在一起。已发现当第一反应(该反应进行以连接生理活性多肽或免疫球蛋白恒定区和非肽基聚合物)在低温下、在低浓度还原剂的存在下进行短时间时,偶联反应的产率提高(表 1)。

[0027] 在另一个优选的实施方式中,用不同浓度的还原剂硼烷吡啶复合物进行反应,并在较低浓度的还原剂用于生理活性多肽或免疫球蛋白恒定区和非肽基聚合物之间的第一反应之后,检测到第二反应(偶联反应)的产率较高。与使用硼烷吡啶复合物的情况相比,使用氰基硼氢化钠作为还原剂的情况显示出更高的产率(表 2)。

[0028] 而且,已经确认第二反应(偶联反应)的产率随着还原剂浓度的升高而增加。

[0029] 在一个优选的实施方式中,以不同浓度的还原剂氰基硼氢化钠进行偶联反应,在高浓度还原剂的存在下得到提高的偶联反应产率。然而,很高浓度的还原剂引起免疫球蛋白恒定区出现异常。为了避免此异常,偶联反应在 20mM 氰基硼氢化钠的存在下进行 13 小时,观察到其产率维持在高水平,而且免疫球蛋白恒定区的异常因此而最小化(表 3 和表 4)。

[0030] 本文所使用的术语“生理活性多肽”是指一般概念上在体内具有某种生理功能的多肽。其共有多肽基结构并显示出各种不同的生物学活性。当由于涉及某种功能的材料缺少或过量而使身体出现生物异常时,生理活性多肽可调节基因表达或生理功能,从而改正该异常。典型的例子是蛋白质药物。

[0031] 应用于本发明的生理活性多肽实例包括人生长激素、生长激素释放激素、生长激素释放肽、干扰素、干扰素受体、集落刺激因子、类胰高血糖素肽(GLP-1,等)、胃泌酸调节素、G 蛋白质-偶联受体、白细胞介素、白细胞介素受体、酶、白细胞介素-结合蛋白、细胞因子-结合蛋白、巨噬细胞活化因子、巨噬细胞肽、B-细胞因子、T-细胞因子、蛋白质 A、变态反应抑制剂、细胞坏死糖蛋白、免疫毒素、淋巴毒素、肿瘤坏死因子、肿瘤抑制剂、转化生长因子、 α -1 抗胰蛋白酶、白蛋白、 α -乳清蛋白、载脂蛋白-E、促红细胞生成素、糖基化促红

细胞生成素、血管生成素、血红素、凝血酶、凝血酶受体活化肽、血栓调节素、血液因子 VII、VIIa、VIII、IX 和 XIII、纤溶酶原激活剂、纤维蛋白 - 结合肽、尿激酶、链球菌激酶、水蛭素、蛋白质 C、C- 反应蛋白、肾素抑制剂、胶原酶抑制剂、超氧化歧化酶、瘦蛋白、血小板 - 衍生生长因子、上皮细胞生长因子、表皮细胞生长因子、血管抑制素、血管收缩素、骨生长因子、骨刺激蛋白、降血钙素、胰岛素、心钠素、软骨诱导因子、依降钙素、结缔组织活化因子、组织因子途径抑制剂、卵泡刺激激素、黄体生成素、黄体生成素释放激素、神经生长因子、甲状旁腺激素、松弛肽、分泌素、生长调节素 (somatomedin)、类胰岛素生长因子、肾上腺皮质激素、胰高血糖素、胆囊收缩素、胰腺多肽、胃泌素释放肽、促肾上腺皮质激素释放因子、甲状腺刺激激素、自毒素、乳铁蛋白、肌肉抑制素、细胞表面抗原、病毒 - 衍生疫苗抗原、单克隆抗体、多克隆抗体和抗体片段。

[0032] 本发明实施方式中所使用的胰岛素是一种分泌自胰腺的生物活性肽，当血糖水平变高时，其作用是通过引起肝脏、骨骼肌和脂肪组织摄取血液中的葡萄糖并将其存储为糖原，并抑制脂类分解（其是利用脂肪作为能量来源的新陈代谢）来控制血糖水平。生物活性肽包括胰岛素激动剂、前体、衍生物、片段和变体。优选天然胰岛素、速效胰岛素和长效胰岛素。

[0033] 天然胰岛素是一种胰腺分泌的激素，并且通过促进细胞对葡萄糖的摄取和抑制脂类分解，在血糖水平的控制中起关键作用。具有调节血糖水平功能的胰岛素是通过一系列过程，从不具有调节血糖水平功能的胰岛素原前体产生。氨基酸序列如下：

[0034] - α 链：

[0035] Gly-Ile-Val-Glu-Gln-Cys-Cys-Thr-Ser-Ile-Cys-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn (SEQ ID NO:1)

[0036] - β 链：

[0037] Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr (SEQ ID NO:2)

[0038] 本文所使用的术语“胰岛素激动剂”是指可结合体内胰岛素受体并表现出与胰岛素相同的生物活性而与胰岛素的结构无关的物质。

[0039] 本文所使用的术语“胰岛素衍生物”是指这样的肽，其功能是控制体内血糖水平，并且其氨基酸序列与天然胰岛素共有至少 80% 同源性。在一些氨基酸残基中可以有化学取代（例如， α -甲基化、 α -羟基化）、缺失（例如，脱氨基作用）或修饰（例如，N-甲基化、糖基化、脂肪酸）。

[0040] 本文所使用的术语“胰岛素片段”是指具有控制体内血糖水平功能的肽，其通过从胰岛素的氨基或羧基末端添加至少一个氨基酸或删除至少一个氨基酸而制成。添加的氨基酸可以是非天然存在的氨基酸（例如，D-氨基酸）。

[0041] 本文所使用的术语“胰岛素变体”是指这样的肽：其氨基酸序列与天然胰岛素的氨基酸序列差异一个或多个氨基酸残基，但具有控制体内血糖水平的功能。

[0042] 此外，分别用于制备胰岛素激动剂、片段和变体的方法可单独地使用或组合使用。例如，用于本发明的胰岛素肽可包括这样的肽：其氨基酸序列与天然胰岛素的氨基酸序列差异一个或多个氨基酸残基，在 N-末端残基具有脱氨基作用，但其具有控制体内血糖水平的功能。

[0043] 本文所使用的术语“免疫球蛋白恒定区”是指缺少轻链和重链的可变区、重链恒定区 1 (C_{H1}) 和轻链恒定区 (C_L) 的免疫球蛋白片段, 即, 由重链恒定区 2 和 3 (C_{H2} 和 C_{H3}) (或包括重链恒定区 (C_{H4})) 构成的 Fc 区。任选地, 免疫球蛋白 Fc 区可进一步包括铰链区。而且, 本发明的免疫球蛋白恒定区可以是只排除免疫球蛋白的重链和轻链的可变区而包括一部分或全部重链恒定区 1 (C_{H1}) 和 / 或轻链恒定区 (C_L) 的延长免疫球蛋白 Fc 区, 只要其显示出与天然免疫球蛋白恒定区实质上相同或更好的效果。进一步, 本发明的免疫球蛋白恒定区可缺少对应于 C_{H2} 和 / 或 C_{H3} 的氨基酸序列的主要部分。因此, 本发明的免疫球蛋白恒定区可包括 (1) C_{H1} 结构域、 C_{H2} 结构域、 C_{H3} 结构域和 C_{H4} 结构域, (2) C_{H1} 结构域和 C_{H2} 结构域, (3) C_{H1} 结构域和 C_{H3} 结构域, (4) C_{H2} 结构域和 C_{H3} 结构域, (5) 一个或多个恒定结构域和免疫球蛋白铰链区 (或部分铰链区) 的组合, 或 (6) 重链的每个恒定结构域和轻链的恒定区的二聚体。

[0044] 包含 Fc 区的免疫球蛋白恒定区是可在体内代谢的生物可降解多肽, 所以其可安全地用作药物载体。此外, 由于分子量相对较低, 免疫球蛋白 Fc 区在复合物的生产、纯化和产率方面均比完整的免疫球蛋白分子更有利。进一步, 由于其缺少 Fab (其因为在抗体与抗体之间的氨基酸序列的差异而表现出高的非同质性), 所以免疫球蛋白 Fc 单独可提供具有显著增强同质性的复合物, 并且降低了诱发血液抗原性的可能性。

[0045] 免疫球蛋白恒定区可来源于人或动物, 例如, 奶牛、山羊、猪、小鼠、兔、仓鼠、大鼠、天竺鼠等, 且优选地为人类来源。此外, 免疫球蛋白恒定区可选自衍生于 IgG、IgA、IgD、IgE、IgM 的 Fc 片段、或其组合或其杂化物。优选地, 恒定区衍生自 IgG 或 IgM (它们是血液中最丰富的), 并且最优选衍生自 IgG (已知其改善配体 - 结合蛋白质的血清半衰期)。

[0046] 本文所使用的术语“组合”意指相同来源的编码单链免疫球蛋白恒定区 (优选 Fc 区) 的多肽连接至不同来源的单链多肽以形成二聚体或多聚体。即, 二聚体或多聚体可通过选自 IgG Fc、IgA Fc、IgM Fc、IgD Fc 和 IgE Fc 片段的两种或更多种片段的组合而制成。

[0047] 本文所使用的术语“杂化物”意指编码不同来源的两种或更多种免疫球蛋白 Fc 片段的序列存在于免疫球蛋白恒定区 (优选地, Fc 区) 的单链中。本发明中, 多种杂化物形式是可能的。例如, 杂化物结构域可由选自 IgG Fc、IgM Fc、IgA Fc、IgE Fc 和 IgD Fc 的 C_{H1} 、 C_{H2} 、 C_{H3} 和 C_{H4} 的一种至四种结构域组成, 并可包括铰链区。

[0048] IgG 被分成 IgG1、IgG2、IgG3 和 IgG4 亚类, 并且本发明可包括其组合或杂化物。优选为 IgG2 和 IgG4 亚类, 并且最优选为几乎没有效应子功能 (例如, 补体依赖的细胞毒性 (CDC)) 的 IgG4 的 Fc 区。

[0049] 免疫球蛋白恒定区可具有糖基化形式, 其糖基化程度可与天然形式相同, 或更高或更低, 或可以为去糖基化形式。通过典型的方法, 例如, 使用化学方法、酶法或使用微生物的基因工程法, 可以实现免疫球蛋白区糖基化或去糖基化的升高或降低。其中, 当去糖基化时, 连接至免疫球蛋白 Fc 区的补体 (C1q) 会显著减少, 并且抗体 - 依赖的细胞毒性或补体 - 依赖的细胞毒性降低或移除, 从而不会引起不必要的体内免疫应答。本文中, 去糖基化或无糖基化免疫球蛋白 Fc 区更符合药物载体的目的。因此, 最适合作为本发明药物载体的免疫球蛋白 Fc 区是人类 IgG4- 衍生的无糖基化 Fc 区。人类 - 衍生的 Fc 区比非 - 人类衍生的 Fc 区更优选, 非 - 人类衍生的 Fc 区可充当人体内的抗原并引发不需要的免疫应答, 例如, 针对抗原的新抗体的产生。

[0050] 而且,本发明的免疫球蛋白恒定区的范围不仅可以包括具有天然氨基酸序列的免疫球蛋白恒定区,而且可以包括其氨基酸序列突变体。本文所使用的术语“氨基酸序列突变体”是指氨基酸序列由于一个或多个氨基酸残基的缺失、插入、保守性或非保守性置换或其组合而不同于野生型的多肽。例如,已知对连接重要的 IgG Fc 中的位置 214 至 238、297 至 299、318 至 322 或 327 至 331 上的氨基酸残基可用作为适于修饰的位置。本发明可使用多种衍生物,例如,通过从天然 Fc 中移除可形成二硫键的位置、移除数个 N-末端氨基酸或将甲硫氨酸添加至天然 Fc 的 N-末端而制备的衍生物。此外,补体固定位置,例如, C1q 固定位置,或 ADCC 位置可从天然 Fc 区消除以移除效应子功能。制备免疫球蛋白恒定区的氨基酸序列突变体的技术已在国际专利公开号 WO 97/34631 和 WO 96/32478 中公开。

[0051] 蛋白质或肽分子中不改变分子活性的氨基酸置换是本领域众所周知的 (H. Neurath, R. L. Hill, The Proteins, Academic Press, New York, 1979)。最常见的置换发生在氨基酸残基 Ala/Ser、Val/Ile、Asp/Glu、Thr/Ser、Ala/Gly、Ala/Thr、Ser/Asn、Ala/Val、Ser/Gly、Thr/Phe、Ala/Pro、Lys/Arg、Asp/Asn、Leu/Ile、Leu/Val、Ala/Glu 和 Asp/Gly 之间。任选地,氨基酸可通过磷酸化、硫酸化、丙烯酸化、糖基化、甲基化、法尼基化、乙酰化和酰胺化进行修饰。

[0052] 上述免疫球蛋白恒定区衍生物表现出与本发明的免疫球蛋白恒定区相同的生物学活性,但其对于热、pH 等具有改进的结构稳定性。这些免疫球蛋白恒定区可从分离自人或动物,例如,牛、山羊、猪、小鼠、兔、仓鼠、大鼠、天竺鼠等的天然型中获得,或可以从转化动物细胞或微生物中得到的重组体或衍生物。可通过对从人类或动物样品中分离的免疫球蛋白的整个范围进行蛋白酶消化得到天然恒定区。免疫球蛋白用木瓜蛋白酶裂解为 Fab 和 Fc,和用胃蛋白酶裂解为 pF' c 和 F(ab)2,然后通过尺寸排阻色谱法从其中分离 Fc 或 pF' c。

[0053] 优选从微生物中获得的重组人免疫球蛋白恒定区。

[0054] 发明的有利效果

[0055] 如上所述,可以利用本发明的方法以高纯度和高产率以及低成本制备生理活性多肽-非肽基聚合物-免疫球蛋白恒定区复合物。因此,本发明的方法适用于工业。而且,其可用于开发具有改善的药物顺应性的生理活性多肽的长效制剂。

具体实施方式

[0056] 通过以下实施例可获得对本发明更好的理解,这些实施例提供说明,但不应解释为对本发明的限制。

[0057] 实施例 1:使用氰基硼氢化钠作为还原剂的胰岛素 PEG 化和单-PEG 化胰岛素的纯化

[0058] 将胰岛素粉末溶于 10mM HCl,并用 3.4K 丙(propion)-ALD2 PEG(具有两个丙醛基的 PEG, IDB, 韩国)在 β 链的 N-末端进行 PEG 化。在此时,使 5mg/ml 胰岛素与 PEG 以 1:2 的摩尔比在 4℃至室温下反应 2 小时。反应在 2-20mM 氰基硼氢化钠作为还原剂的存在下,在 45%异丙醇中的 50mM 柠檬酸钠缓冲液(pH 6.0)中进行。将反应混合物装载到 SP-HP (GE Healthcare) 柱上,然后用含柠檬酸钠(pH 3.0)和 45% EtOH 的缓冲液洗脱并使用 KCl 的浓度梯度纯化单-PEG 化胰岛素。

[0059] 在制备含胰岛素和免疫球蛋白 Fc 区的复合物期间,根据还原剂氰基硼氢化钠的

条件,胰岛素的 PEG 化产率总结于下表 1。

[0060] 实施例 2:根据 PEG 化中用作还原剂的氰基硼氢化钠的条件,单-PEG 化胰岛素-免疫球蛋白 Fc 区复合物产率的变化

[0061] 为了检测胰岛素-PEG-免疫球蛋白 Fc 区复合物的产率,使实施例 1 中制备的单-PEG 化胰岛素与免疫球蛋白 Fc 以摩尔比 1:1 在 25℃下反应 13 小时,总蛋白质浓度设置为 20mg/ml。该偶联反应在 20mM 氰基硼氢化钠作为还原剂的存在下,在 100mM HEPES 缓冲液(含 22mM 磷酸钾和 10%乙醇, pH 8.2)中进行。

[0062] 将反应混合物装载到 Source 15Q(GE Healthcare)柱上,然后用 Tris-HCl(pH 7.5)缓冲液洗脱并使用 NaCl 的浓度梯度分离和纯化未反应的胰岛素、未反应的免疫球蛋白 Fc 区、胰岛素-PEG-免疫球蛋白 Fc 区复合物和与两个或更多个单-PEG 化胰岛素(胰岛素-PEG)部分偶联的免疫球蛋白 Fc 区。通过色谱法纯化后,在 280nm 的 UV 吸光度下测定胰岛素-PEG-免疫球蛋白 Fc 区复合物的产率。

[0063] 表 1 中,根据胰岛素 PEG 化中用作还原剂的氰基硼氢化钠的条件,总结了与免疫球蛋白 Fc 区偶联反应的产率。

[0064] 【表 1】

[0065]

氰基硼氢化钠的浓度	反应时间	反应温度	PEG 化产率(%)	偶联产率(%)	总产率(%)
2 mM	2hr	4℃	23.3	31.7	7.39
4 mM	2hr	4℃	37.6	31	11.66
4 mM	2hr	RT	39.2	28.8	11.29

[0066]

8 mM	2hr	RT	40.4	27.1	10.95
8 mM	4hr	4℃	40.4	27	10.9
20 mM	2hr	4℃	42.2	26.8	11.3

[0067] 实施例 3:使用硼烷吡啶复合物作为还原剂的胰岛素 PEG 化和单-PEG 化胰岛素的纯化

[0068] 将胰岛素粉末溶于 10mM HCl,并用 3.4K 丙-ALD2 PEG(具有两个丙醛基的 PEG, IDB, 韩国)。在此时,使 5mg/ml 胰岛素与 PEG 以摩尔比 1:2 在 4℃下反应 2 小时。该反应在 3 至 20mM 硼烷吡啶复合物作为还原剂的存在下,在 45%异丙醇中的 50mM 柠檬酸钠缓冲液(pH 6.0)中进行。将反应混合物装载到 SP-HP(GE Healthcare)柱上,然后用含柠檬酸钠(pH 3.0)和 45% EtOH 的缓冲液洗脱并使用 KCl 的浓度梯度纯化单-PEG 化胰岛素。

[0069] 在制备含胰岛素和免疫球蛋白 Fc 区的复合物期间,根据还原剂硼烷吡啶复合物的条件,胰岛素的 PEG 化产率总结于下表 2。

[0070] 实施例 4:根据 PEG 化中作为还原剂使用的硼烷吡啶复合物的条件,单-PEG 化胰岛素-免疫球蛋白 Fc 区复合物产率的变化

[0071] 为了检测胰岛素-PEG-免疫球蛋白 Fc 区复合物的产率,使实施例 3 中制备的

单-PEG 化胰岛素与免疫球蛋白 Fc 以 1:1 的摩尔比在 25℃ 下反应 13 小时,总蛋白质浓度设置为 20mg/ml。该偶联反应在 20mM 氰基硼氢化钠作为还原剂的存在下,在 100mM HEPES 缓冲液(含 22mM 磷酸钾和 10%乙醇, pH 8.2)中进行。

[0072] 将反应混合物装载到 Source 15Q(GE Healthcare)柱上,然后用 Tris-HCl(pH 7.5)缓冲液洗脱并使用 NaCl 的浓度梯度分离和纯化未反应的胰岛素、未反应的免疫球蛋白 Fc 区、胰岛素-PEG-免疫球蛋白 Fc 区复合物和与两个或更多个单-PEG 化胰岛素(胰岛素-PEG)部分偶联的免疫球蛋白 Fc 区。通过色谱法纯化后,在 280nm 的 UV 吸光度下测定胰岛素-PEG-免疫球蛋白 Fc 区复合物的产率。

[0073] 表 2 中,根据胰岛素 PEG 化中用作为还原剂的硼烷吡啶复合物的条件,总结了与免疫球蛋白 Fc 区偶联反应的产率。

[0074] 【表 2】

[0075]

硼烷吡啶复合物的浓度	PEG 化产率 (%)	偶联产率 (%)	总产率 (%)
3 mM	25.4	35.1	8.92
10 mM	47.6	34.8	16.6
20 mM	50.8	34.2	17.4

[0076] 实施例 5:根据氰基硼氢化钠的浓度和反应时间,偶联反应的产率和免疫球蛋白 Fc 异常的形成

[0077] 根据偶联反应中还原剂的浓度和反应时间,为了检测免疫球蛋白 Fc 异常的形成,使单-PEG 化胰岛素与免疫球蛋白 Fc 以摩尔比 1:1 在 25℃ 下反应 13 至 43 小时,总蛋白质浓度设置为 20mg/ml。该偶联反应在 5 至 40mM 氰基硼氢化钠的存在下,在 100mM HEPES 缓冲液(含 22mM 磷酸钾和 10%乙醇, pH 8.2)中进行。

[0078] 将反应混合物装载到 Source 15Q(GE Healthcare)柱上,然后用 Tris-HCl(pH 7.5)缓冲液洗脱并使用 NaCl 的浓度梯度分离和纯化未反应的胰岛素、未反应的免疫球蛋白 Fc 区、胰岛素-PEG-免疫球蛋白 Fc 区复合物和与两个或更多个单-PEG 化胰岛素(胰岛素-PEG)部分结合的免疫球蛋白 Fc 区。通过色谱法纯化后,在 280nm 的 UV 吸光度下测定胰岛素-PEG-免疫球蛋白 Fc 区复合物的产率。

[0079] 表 3 中,根据偶联反应中用作还原剂的氰基硼氢化钠的浓度和反应时间,总结了用来制备含胰岛素和免疫球蛋白 Fc 区复合物的偶联反应的产率。

[0080] 【表 3】

[0081]

反应时间	5mM SCB	20mM SCB	40mM SCB
13 小时	35.2%	37.5%	37.5%
18 小时	36.1%	37.7%	37.7%
37 小时	36.7%	37.3%	37.5%

43 小时	36.8%	37.2%	36.8%
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[0082] 通过 Propac SAX-10 (DI 一种 X) 柱上的 LC 以 Tris-HCl (pH 8.0) 缓冲液洗脱并使用 NaCl 的浓度梯度, 根据偶联反应中还原剂的浓度, 监测免疫球蛋白 Fc 异常的形成。

[0083] 表 4 中, 根据用来制备含胰岛素和免疫球蛋白 Fc 区复合物的偶联反应中氰基硼氢化钠的浓度和反应时间, 给出了免疫球蛋白 Fc 异常的产率。

[0084] 【表 4】

[0085]

反应时间	5mM SCB	20mM SCB	40mM SCB
13 小时	4.6%	7.0%	7.8%
18 小时	6.2%	9.0%	9.8%
37 小时	11.7%	14.5%	15.3%
43 小时	12.7%	15.5%	16.8%

[0086] 实施例 6 : 使用氰基硼氢化钠作为还原剂的免疫球蛋白区 Fc 的 PEG 化和单 -PEG 化免疫球蛋白 Fc 的纯化

[0087] 用 5K 丙 -ALD2 PEG (具有三个丙醛基的 PEG, NOF, 日本) 将免疫球蛋白 Fc 的 N- 末端进行 PEG 化。在此时, 使 10mg/ml 免疫球蛋白 Fc 与 PEG 以摩尔比 1:2 在 4℃ 至室温下反应 4.5 小时。该反应在 20mM 氰基硼氢化钠作为还原剂的存在下, 在 100mM 磷酸钾缓冲液 (pH 6.0) 中进行。将反应混合物装载到 Source 15Q (GE Healthcare) 柱上, 然后用 Tris-HCl (pH 7.5) 缓冲液洗脱并使用 NaCl 的浓度梯度以纯化单 -PEG 化胰岛素。

[0088] 实施例 7 : 使用氰基硼氢化钠作为还原剂的单 -PEG 化免疫球蛋白 Fc 区 - 胰岛素复合物的制备

[0089] 为了制备胰岛素 -PEG- 免疫球蛋白 Fc 区复合物, 使实施例 6 中制备的单 -PEG 化免疫球蛋白 Fc 与胰岛素以摩尔比 1:4 在 4℃ 下反应 13 小时, 总蛋白质浓度设置为 20mg/ml。该偶联反应在 20mM 氰基硼氢化钠作为还原剂的存在下, 在 100mM 磷酸钾缓冲液 (pH 6.0) 中进行。

[0090] 将反应混合物装载到 Source 15Q (GE Healthcare) 柱上用于初次纯化。并且用 Source 15IS0 (GE Healthcare) 柱另外进行二次纯化, 以获得胰岛素 -PEG- 免疫球蛋白 Fc 区复合物。

[0001]

序列表

<110> 韩美科学株式会社

<120> 制备生理活性多肽复合物的改进方法

<130> OPA13018

<150> KR 10-2012-0024136

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<160> 2

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<212> PRT

<213> 智人

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1				5				10						15	

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

An Improved Process for Preparation of Physiologically Active Polypeptide Complex

ABSTRACT

Disclosed is a method for the preparation of a complex in which a physiologically active polypeptide is covalently bonded to an immunoglobulin constant region via a non-peptidyl linker. The method is characterized by the employment of a reducing agent, by which conventional problems of low production yield and modification of the polypeptide can be overcome. The physiologically active polypeptide-non-peptidyl polymer-immunoglobulin constant region complex can be produced with high purity and yield as well as at low cost. Thus, the method is industrially useful. Moreover, by exhibiting a prolonged action profile, the physiologically active polypeptide-non-peptidyl polymer-immunoglobulin constant region complex can be effectively used for developing long-acting formulations of physiologically active polypeptides which have improved drug compliance.

制备生理活性多肽复合物的改进方法

摘要

本发明公开的是一种制备复合物的方法，该复合物中的生理活性多肽通过非肽基连接体共价结合至免疫球蛋白恒定区。该方法的特征在于还原剂的使用，由此可以克服传统的低产率和多肽修饰的问题。可以以高纯度和高产率以及低成本生产生理活性多肽-非肽基聚合物-免疫球蛋白恒定区复合物。因此，该方法在工业上是有用的。而且，通过表现出长效特征，该生理活性多肽-非肽基聚合物-免疫球蛋白恒定区复合物可以有效地用于开发具有改善的药物顺应性的生理活性多肽的长效制剂。