# 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, angiopoeitins, 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.

# 【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 from more than 0 mM to less than 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 from more than 0 mM to less than 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, angiopoeitins, 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.

【Claim 24】

The method of claim 1, wherein the reducing agent of the step (1) is used in a concentration of from more than 0mM to 10mM or less than 10mM.

【Claim 25】

The method of claim 1, wherein the reducing agent of the step (1) is used in a concentration of from more than 0mM to 8mM or less than 8mM.

【Claim 26】

The method of claim 1, the yield of the coupling reaction is increased compared to when the reducing agent in step (1) is at a concentration of 20mM or higher.