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REF:

Expediente 13-009-151

TÍTULO SOLICITADO:

**“UN NUEVO CONJUGADO DE GRANULOCITO
COLONIA-ESTIMULANTE FACTOR (G-CSF) CON
POLIETILENO”**

PUBLICACIÓN:

**Gaceta 667 de 15 de mayo de 2013, N° de
publicación 557.**

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SOLICITANTE:

CLOSED JOINT STOCK COMPANY BIOCAD

APODERADO:

JAIME EDUARDO DELGADO VILLEGAS

TRÁMITE:

SUSTENTO DE OPOSICIÓN

JOSÉ LUIS REYES VILLAMIZAR, mayor de edad, identificado como aparece al pie de mi firma, abogado en ejercicio, titular de la tarjeta profesional número 44655 del Consejo Superior de la Judicatura, obrando en calidad de apoderado de la sociedad **TECNOQUIMICAS S.A.**, dentro del trámite de la referencia, en ejercicio de lo dispuesto por el artículo 42 inciso 2° de la Decisión 486 de 2000, por medio del presente escrito, procedo a sustentar y complementar la oposición presentada el 12 de agosto de 2013, en los siguientes términos:

I. RATIFICACIÓN

La solicitud 13-009-151 referida a conjugados del factor estimulante de colonia de granulocitos, carece de Claridad, Novedad y Nivel Inventivo, de acuerdo a la materia técnica revelada por el documento US 5824784 de 20/10/1998.

En primer lugar, se objetó la claridad, concisión y suficiencia de la descripción y reivindicaciones de la solicitud en trámite, dado que el polipéptido reclamado no está adecuadamente definido en las reivindicaciones ya que la formula general reclamada presenta una amplitud tal, que permite reclamar cadenas de longitud indeterminada sin tener una probabilidad razonable de cumplir con las características funcionales y estructurales divulgadas.

Se observó además que la materia es ambigua y poco clara en la naturaleza del péptido al no presentarse definición del origen, lo cual da como resultado una variación que incluso pertenece a materia no patentable al tratarse de polipéptidos de origen natural.

Las reivindicaciones 6 y 8 por su parte, no definen claramente la materia técnica sino que se caracterizan por parámetros y medidas no determinadas y las reivindicaciones 9 y 10 no mencionan características determinantes de las composiciones reivindicadas.

En cuanto a la materia revelada en las reivindicaciones 13 y 7 relacionada con el uso del conjugado y el uso de las composiciones farmacéuticas, no son patentables de acuerdo a lo contemplado en la Decisión 486.

En adición a las objeciones ya planteadas, se observó que las reivindicaciones 14, 11 y 12 se refieren a un método de tratamiento, exceptuado de patentabilidad, motivo por el cual la solicitud y en especial dichas reivindicaciones no pueden ser concedidas.

Por su parte, el documento US 5824784 se destaca por contener *la materia relevante* de las reivindicaciones debido a que este documento describe un conjugado del factor estimulante de colonia de granulocitos (G-CSF) PEGilado, que tiene diferentes modificaciones en la molécula de polietileno.

La determinación de moléculas específicas de PEG unidas a G-CSF son un aspecto rutinario para el técnico versado en la materia, en la medida en que no se requiere actividad inventiva para determinar la longitud de la cadena, según lo revelado por lo anterioridad.

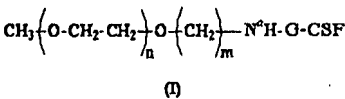
También se describieron métodos de conjugación conocidos en la molécula de G-CSF para obtener mono y poli isómeros PEGilados, donde incluso la molécula de polietilen glicol se encuentra sustituido en el ejemplo 2, con monometoxipolietileno glicol que es empleado en la fórmula 1 de la solicitud en estudio.

En consecuencia, un técnico versado en la materia a la luz del documento US5824784, habría llegado a los conjugados reclamados por la presente solicitud 13 009-151 pues ya se revelaban conjugados de factor estimulante de colonia de granulocitos (G-CSF) y monometoxipolietilen glicol, dentro de los parámetros de la formula mencionada por la solicitud colombiana.

II. COMPLEMENTO DE INFORMACIÓN

De acuerdo con la reivindicación 1 de la solicitud en trámite, existen diferentes documentos del estado de la técnica que mencionan las características esenciales que se reivindican tal como es el caso de un conjugado de PEG con hG-CSF, (dicha reivindicación se cita a continuación para efectos de comparación entre los documentos):

1. Una colonia estimulante humana granulocita factor conjugado con monometoxyplietileno glicol de fórmula (1).



Donde:

n – intergers de 681 a 1 000;
m – interger ≥ 4;
N^HH-G-CSF – Natural o recombinante polipéptido, teniendo actividad de G-CSF

La publicación internacional WO 2004/083242 por ejemplo, menciona un conjugado derivado de hG-CSF modificado con un PEG, el cual muestra un aumento en la estabilidad y vida media prolongada en la sangre mientras reduce la posibilidad de inducir una respuesta inmune.

25 Summary of the Invention

Accordingly, a primary object of the present invention is to provide a PEG conjugate of an hG-CSF derivative having a prolonged *in vivo* half-life and an improved biological activity without inducing an immune response in a
30 subject; and a method for the preparation thereof.

Si tenemos en cuenta que las características estructurales que se pretenden proteger en la solicitud en trámite son: 1) una colonia estimulante humana granulocito factor (hG-CSF); 2) el monometoxipolietileno glicol y 3) la fórmula sobre la longitud de dicho monometoxipolietileno, dichas características son anticipadas por la anterioridad en mención y como tal, en su ejemplo 2 la preparación incluye el conocimiento de los tres factores esenciales para la preparación del conjugado, veamos:

Example 2: Preparation of PEG-hG-CSF derivative conjugate

5

HM10411 was dissolved in 100 mM potassium phosphate buffer (pH 6.0) to a concentration of 5 mg/ml, distributed into three vials, and then, adjusted to a final volume of 5 ml. An activated methoxy-PEG-aldehyde (Shearwater, USA) which has 20 kDa or 40 kDa of PEG was added to each of
10 two vials in an amount corresponding to a HM10411:methoxy-PEG-aldehyde molar ratio of 1:4. Sodium cyanoborohydride (NaCNBH₃, Sigma, USA) was added thereto to a final concentration of 20 mM, and the reaction mixture was stirred at 4°C for 18 hrs. Then, ethanolamine was added thereto to a final concentration of 50 mM to inactivate unreacted PEG.

15

To further remove unreacted PEG, the reaction mixture was subjected to Sephadex G-25 column chromatography (Pharmacia). Namely, after the column was equilibrated with 2 CV (column volume) of 10 mM Tris-HCl buffer (pH 7.5), the reaction mixture was loaded onto the column and eluted with the same buffer. Elution fractions were analyzed for absorbance at 260 nm using
20 a UV spectrophotometer. As a result, the PEG modified HM10411 which has a large molecular weight was eluted before unreacted PEG.

Estas características estructurales son las que permiten en este caso, demostrar el incumplimiento a los requerimientos de patentabilidad por parte de la solicitud en trámite, adicionalmente, las demás características o características funcionales obedecen a un resultado a lograr de las características estructurales y no definen la materia, tal como la actividad presente en el conjugado.

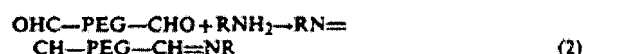
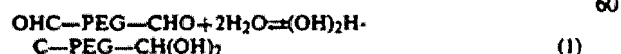
- En cuanto a las características modeladas en la fórmula, incluyen únicamente un alargamiento no especificado en la molécula de polietilenglicol o monometoxipolietileno, con lo cual, cualquier molécula en un tamaño razonablemente empleado en la técnica cumple las características planteadas por la solicitud.

De otro lado, existen otros documentos que describen la misma solución al problema técnico de la solicitud en trámite, tal es el caso de la patente americana US 5,252,714 ya que en ella se revelan derivados de PEG con características como las de no ser destruido por agua, ser capaces de retener actividad en agua y reaccionar selectivamente con grupos aminos.

Este documento menciona que es de uso normal el empleo de Propionaldehído o equivalentes de PEG para el desarrollo de conjugados tal como se hace en la Reivindicación 1 ya que se habla de derivados de PEG, veamos:

USE OF PEG PROPIONALDEHYDE OR EQUIVALENTS

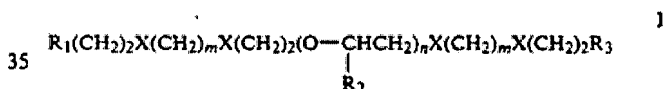
The prime characteristic of the present novel derivative I is that it is not destroyed by water. As shown in equations 1 and 2, compound I exists in water in equilibrium with its hydrate, and it reacts readily with amino groups present on other molecules. The imine that forms can be further reduced (e.g., with sodium cyanoborohydride or sodium borohydride) to give an amine.



This property of retention of reactivity in water is not possessed by any other active PEG derivative. This property is an extremely valuable one since it permits

En este caso, la reivindicación 1 del documento US 5,252,714 describe una fórmula que presenta sustituciones posibles en radicales donde la sustitución de R2 por H, X por O y R3 incluyendo una proteína tipo anticuerpo, cubija todas las características presentes en el conjugado de la solicitud; a continuación se observan las características presentes en dicho documento:

1. A compound, as described by formula (I):



wherein R₁ and R₃ each independently consist of an element selected from the group consisting of



R₄—N=CH, and R₄—NH—CH₂, wherein R₄ is an antibody; and wherein X is selected from elements of the group consisting of O, and S; and wherein R₂ is a C₁ to C₆ alkyl, or H, and;

wherein n is an integer, and n less than 10,000, and m is an integer selected from the group consisting of 2, 3, 4, 5, or 6.

Es decir que la fórmula reclamada de la anterioridad, incluye las características de la reivindicación 1 de la solicitud en trámite, al mencionar la estructura general del conjugado de proteína y un derivado de PEG que, según las sustituciones se encuentra incluida en la anterioridad. Por lo tanto, este documento resuelve el mismo problema técnico de forma general, con la única diferencia de no mencionar específicamente que la proteína es el factor estimulante de colonia de granulocitos humano, lo cual no obedece a un salto cuantitativo sobre el estado de la técnica ni presenta un efecto sorprendente sobre los conjugados ya citados.

Este documento además describe la pegilación, la cual puede dar en 1 y 1000 del valor de n, el cual es análogo en ambos casos e incluye los valores de n de la solicitud en estudio:

4. A compound, as recited in any one of claims 1, or 2, wherein n is between 10 and 1,000.

Otro documento que menciona específicamente metoxipolietilenglicol como un solvente orgánico farmacéuticamente aceptable es la publicación internacional WO2007/084460 que divulga composiciones biodegradables poliméricas que comprenden agentes peptídicos con estabilidad mejorada y un sistema de liberación controlada que emplea básicamente los componentes del conjugado:

25 [0014] The pharmaceutically acceptable organic solvents may be selected from a group consisting of N-methyl-2-pyrrolidone, methoxypolyethylene glycol, alkoxypolyethylene glycol, polyethylene glycol esters, glycofurol, glycerol formal, methyl acetate, ethyl
30 acetate, methyl ethyl ketone, dimethylformamide, dimethyl sulfoxide, tetrahydrofuran, caprolactam, decylmethysulfoxide, benzyl benzoate, ethyl benzoate, triacetin, diacetin, tributyrin, triethyl citrate, tributyl citrate, acetyl triethyl citrate, acetyl
35 tributyl citrate, triethylglycerides, triethyl phosphate, diethyl phthalate, diethyl tartrate, ethyl

Ahora bien, en cuanto a las proteínas presentes en el conjugado y que se incluyen desde la citada anterioridad, se observa que la reivindicación del factor de estimulación de colonia de granulocitos ha sido incluido en la materia de estudio:

20 angiotensins, thyrotropin releasing hormone (TRH), tumor necrosis factor (TNF), parathyroid hormone (PTH), nerve growth factor (NGF), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony
stimulating factor (GM-CSF), macrophage-colony
25 stimulating factor (M-CSF), heparinase, vascular endothelial growth factor (VEG-F), bone morphogenic

De igual manera, la anterioridad en mención sugiere al técnico versado el empleo de polietilenglicol para modificar los agentes peptídicos de acuerdo a protocolos normales que incluyen los grupos metoxi y sus derivados, tal como se hace en la literatura, la cual encierra estas modificaciones:

[0040] The term "pegylation" used herein refers to
20 the covalent conjugation of a soluble polyethylene glycol to the peptide agents. Polyethylene glycol can be prepared according to standard protocols with one end capped as with a methoxy group and the other end activated for facile conjugation to active groups on
25 peptide agents. For examples, various methods for preparing polyethylene glycols and their use for pegylations are described in the art: [e.g., Roberts MJ, Bentley MD, Harris JM, Chemistry for peptide and protein PEGylation. Adv Drug Deliv Rev. 2002 Jun 17; 54(4): 459-
30 76. Veronese FM. Peptide and protein PEGylation: a review of problems and solutions. Biomaterials. 2001 Mar; 22(5): 405-17 and US Patents Nos. 6,113,906; 5,446,090; 5,880,255], which are all incorporated herein by reference.

Como se observa, las características técnicas de la solicitud en trámite se incluyen en los citados documentos, describiendo las formas de llegar a lo reivindicado y las fórmulas que evidentemente para el técnico versado en la materia, incluyen los compuestos reclamados.

Por lo anterior, los conjugados reclamados en la presente solicitud carecen de novedad y de un efecto técnico sorprendente que soporte el nivel inventivo de los mismos.

III **PRUEBAS**

- El documento US5824784 titulado "*N-Terminally chemically modified protein compositions and methods*" del 20 de octubre de 1998.
- El documento WO 2004/083242, "Human granulocyte-colony stimulating factor conjugate having enhanced stability in blood and process for the preparation thereof" 2006. Páginas 1-2, 6-7 y 8 - 12.
- El documento US 5,252,714 "Preparation and use of polyethylene glycol propionaldehyde" publicado el 12 de octubre de 1993. Todo el documento.
- El documento WO 2007/084460 "Pharmaceutical compositions with enhanced stability". Páginas 1, 6-13, 17 y 19.

Señor Superintendente,



JOSÉ LUIS REYES VILLAMIZAR

C.C. 79'152.473 de Usaquén

T.P.A. 44.655 del C.S.J.



US005252714A

United States Patent [19]

[11] Patent Number: 5,252,714

Harris et al.

[45] Date of Patent: Oct. 12, 1993

- [54] PREPARATION AND USE OF
POLYETHYLENE GLYCOL
PROPIONALDEHYDE
- [75] Inventors: J. Milton Harris; M. R.
Sedaghat-Herati, both of Huntsville,
Ala.
- [73] Assignee: The University of Alabama in
Huntsville, Huntsville, Ala.
- [21] Appl. No.: 618,882
- [22] Filed: Nov. 28, 1990
- [51] Int. Cl.⁵ C07K 3/08; C07C 321/14;
C07C 47/12; C07C 43/10
- [52] U.S. Cl. 530/391.9; 106/287.23;
106/287.24; 106/287.25; 525/515; 530/391.7;
530/406; 530/410; 568/41; 568/494; 568/613
- [58] Field of Search 424/85.8; 530/350, 387,
530/389, 402, 812, 815, 391.7, 391.9, 406, 410;
514/2, 12; 526/273, 289; 568/41, 494, 613, 623,
57; 528/421, 380; 106/287.23, 287.24, 287.25,
287.26, 287.32; 525/512, 513, 515

- [56] References Cited
- U.S. PATENT DOCUMENTS
- | | | | |
|-----------|---------|-------------------|---------|
| 4,002,531 | 1/1977 | Roger | 435/188 |
| 4,029,765 | 6/1977 | Hetting | 424/92 |
| 4,101,380 | 7/1978 | Rubinstein et al. | 530/410 |
| 4,179,337 | 12/1979 | Davis et al. | 435/181 |
| 4,791,192 | 12/1988 | Nakagawa et al. | 530/406 |
| 5,080,891 | 1/1992 | Saifer et al. | 435/180 |
- OTHER PUBLICATIONS
- Veronese et al., J. Pharm. Pharmacol., vol. 35, pp. 757-758 (1983).
- Primary Examiner—Jeffrey E. Russel
Attorney, Agent, or Firm—Oblon, Spivak, McClelland,
Maier & Neustadt

- [57] ABSTRACT
- A new polyethylene glycol derivative (PEG) having the property of not being destroyed by water and also being able to retain reactivity in water and selectively react with amine groups. Also provided is a process for modifying organic or polymer surfaces in water by connecting PEG derivatives to exposed amine groups which provides a process whereby there is efficient linking to organic or polymer surfaces in water by use of the PEG derivatives.
- 19 Claims, No Drawings

PREPARATION AND USE OF POLYETHYLENE GLYCOL PROPIONALDEHYDE

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates generally to polyethylene glycol derivatives (PEGs), their fabrication and use, and more particularly to their use in aqueous environments and even more particularly to their use in aqueous environments to modify other substances such as proteins or surfaces.

2. Description of the Background

PEGs are neutral, hydrophilic polymers that are also soluble in water and a variety of organic solvents, and which possess a wide array of biomedical and biotechnical applications. Once in aqueous solution they are heavily hydrated, highly mobile and exclude other polymers (including proteins and nucleic acids). As a consequence PEGs are nontoxic, nonimmunogenic, and capable of forming aqueous two-phase systems with a variety of polymers. Molecules coupled to PEG become nontoxic, nonimmunogenic, soluble in water and many organic solvents, and surfaces modified by PEG attachment become hydrophilic and protein rejecting. These properties have led to a variety of biotechnical and biomedical applications including: aqueous two-phase partitioning, protein (i.e., enzyme, antibody, antigen) immobilization, drug modification, and preparation of protein rejecting surfaces. In addition, PEG-coated surfaces can be used to control wetting and electroosmosis. The properties of PEG mentioned above are described in the following papers which are hereby incorporated by reference: B. J. Herren, S. G. Slater, J. M. Van Alstine, J. M. Harris, and R. S. Snyder, *J. Colloid Interface Sci.*, 51, 46-55 (1987); and J. M. Harris, D. E. Brooks, J. F. Boyce, R. S. Snyder, and J. M. Van Alstine, in "Dynamic Aspects of Polymer Science" J. D. Andrade, Ed., Plenum 1988 pp. 111-119.

Preparation of new activated PEGs has become central to many studies on PEG applications as shown by J. M. Harris in *J. Macromol. Sci.-Rev. Macromol. Chem. Phys.*, C25, 325-373 (1983) which is hereby incorporated by reference. Typically, useful PEGs contain at least one electrophilic center available for reaction with nucleophilic centers of biomolecules (e.g., lysine, cysteine and like residues of proteins) or surfaces (e.g., aminated glass). A variety of active PEGs have been prepared, yet there is continued interest in synthesis of new derivatives possessing properties not now available. A property of particular interest is increased selectivity, including relative inertness toward water and high reactivity toward desired functional groups. As an example, PEG tresylate, a much used derivative, is reactive toward a variety of nucleophiles including water, while PEG aldehydes are inert toward water and react primarily with amines. Inertness toward water is desired, not only because of efficiency of storage, preparation, and application, but also because it permits stepwise linkage, in aqueous media, of molecules to surfaces and molecules to molecules.

Preparation of activated and selective PEGs mentioned above are provided in the following references which are hereby incorporated by reference: K. Yoshinaga and J. M. Harris, *J. Bioact. Comp. Polym.*, 1, 17-24 (1989); K. Nilsson and K. Mosbach, *Methods in Enzymology*, 104, 56 (1984); C. Delgado, G. E. Francis, and D. Fisher, in "Separations Using Aqueous Phase

Systems," D. Fisher and I. A. Sutherland, Eds., Plenum, London, 1989, pp. 211-213; M.-B. Stark and J. K. Holmberg, *Biotech. Bioeng.*, 34, 942 (1989); J. M. Harris and K. Yoshinaga, *J. Bioact. Comp. Polym.*, 4, 281 (1989); H. Walter, D. E. Brooks, and D. Fisher (Editors), "Partitioning in Aqueous Two-Phase Systems," Academic Press, Orlando, Fla., 1985; D. Fisher and I. A. Sutherland (Editors), "Separations Using Aqueous Phase Systems: Applications in Cell Biology and Biotechnology," Plenum, London, 1989.

U.S. Pat. No. 4,002,531 (1977) describes a preparation of PEG acetaldehyde for attaching PEG to enzymes and other proteins. However, the subsequently published article by Harris mentioned above, indicates that the preparation as described in the U.S. Pat. No. 4,002,531 gives very little of the desired aldehyde. More importantly, PEG acetaldehyde is unstable in the presence of base and of limited utility for protein or surface modification in water as shown by M. S. Paley and J. M. Harris, in *J. Polym. Sci. Polym. Chem. Edn.*, 25, 2447-2454 (1987).

U.S. Pat. No. 4,179,337 (1979) to Davis et al indicates that PEG can be attached to proteins (such as enzymes and insulin) to provide PEG-protein conjugates which are soluble and nonimmunogenic. Several uses for these nonimmunogenic PEG-proteins are described and claimed. Also a wide variety of methods for attaching PEG to proteins to provide soluble PEG-protein conjugates are described and claimed. Each of the Davis et al methods for attaching PEG to proteins uses conventional, water-sensitive PEGs.

SUMMARY OF THE INVENTION

An object of this invention is to provide a new PEG derivative which is not destroyed by water, retains reactivity in water, and selectively reacts with amine groups.

Another object of this invention is to provide a process for modifying organic or polymer surfaces in water by connecting PEG derivatives to exposed amine groups.

Yet another object of this invention is to provide a process for efficiently linking or tethering molecules to organic or polymer surfaces in water by use of PEG derivatives.

Yet another object of the invention is to provide a PEG derivative which selectively reacts with amine groups.

These objects of the invention are achieved by preparing and using a novel compound or its chemical equivalents: PEG Propionaldehyde. PEG propionaldehyde is stable in water and selectively reacts with amine groups in water. The reaction of the amine and the aldehyde produces an imine which can easily be reduced to form a stable amine bond. PEG propionaldehyde reacted in such a manner maintains a second aldehyde group away from the amine containing site or surface. This second aldehyde group can be used to tether another amine containing material.

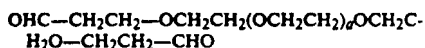
The ideal PEG derivative is: (1) reactive with nucleophilic groups (typically amino) on proteins and surfaces; (2) stable in aqueous media and on the shelf; (3) easily prepared and characterized; and (4) capable of coupling to proteins without reducing protein activity. Many of these properties are potentially possessed by PEG aldehydes. In particular, aldehydes are in equilibrium with their hydrates in water, and will react in this medium

with amines to form imines (which can be reduced in situ with sodium cyanoborohydride). The availability of aqueous coupling chemistry has several benefits. For example, surfaces can be modified that either dissolve or soften deleteriously in organic solvents. Secondly, PEG aldehydes can potentially be used for tethering molecules to surfaces in water. To do this, PEG can be first coupled to the surface, in water, and then reacted in a second step still in water, to some other molecule such as a protein. Previous activated PEGs cannot be used in this fashion since they react with water too rapidly to permit the two-step process. A second potential benefit is that the aldehyde reacts only with amino groups and not with other nucleophilic centers.

As a consequence, synthesis of PEG aldehydes has been actively pursued. As a first attempt PEG acetaldehyde (PEG-O-CH₂CHO) was prepared. This compound has proven useful in some cases and is the subject of the Royer patent mentioned above, however reproducible results are difficult to achieve. Closer examination showed that the compound is very sensitive to decomposition in aqueous base, presumably by aldol condensation. If aldol condensation is the decomposition route then more desirable derivatives would be the benzaldehyde derivative and the propionaldehyde derivative. The benzaldehyde derivative was prepared but it proved too unreactive to be of general use.

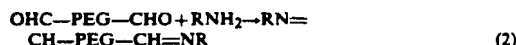
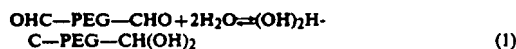
DESCRIPTION OF THE PREFERRED EMBODIMENTS

A new PEG aldehyde (i.e., PEG propionaldehyde or: I) possessing unique properties is presented. This novel PEG fits into a class of PEG derivatives which could be described as "active" PEGs because they are chemically active and ready for chemical attachment to other molecules and surfaces. There are many active PEGs, and their prime applications are in attachment of PEG to proteins and surfaces to alter the properties of the proteins and surfaces in a desirable way. Shown below are the formula for PEG and PEG propionaldehyde:



USE OF PEG PROPIONALDEHYDE OR EQUIVALENTS

The prime characteristic of the present novel derivative I is that it is not destroyed by water. As shown in equations 1 and 2, compound I exists in water in equilibrium with its hydrate, and it reacts readily with amino groups present on other molecules. The imine that forms can be further reduced (e.g., with sodium cyanoborohydride or sodium borohydride) to give an amine.



This property of retention of reactivity in water is not possessed by any other active PEG derivative. This property is an extremely valuable one since it permits

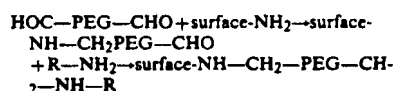
chemical modification of other substances in water without concern for loss of activity during reaction. It should be noted that it is frequently necessary to conduct these modification procedures in water because the substance to be modified (e.g., a protein or an organic polymer such as a plastic) may be harmed by a water-free organic solvent. At present, other active but water-sensitive PEGs are used for reaction in water with the understanding that a significant loss of active PEG will result. As described below, this loss of activity has serious consequences.

Two applications serve to illustrate the value of retention of chemical reactivity in water.

First, this property means that one can modify other substances (such as proteins or surfaces) in water with great efficiency (an economic benefit); i.e., no by-product is produced from reaction of activated PEG with water.

Secondly, water stability means that this active PEG I can be used to link or tether molecules to organic-polymer surfaces in water. Several works have shown that PEG is an unusually effective linker for tying active molecules onto surfaces (see for example references by H. A. Jacobs, T. Okano, and S. W. Kim in *J. Biomed. Mat. Res.*, 23, 611 (1989); S. Nagaoka, H. Murumatani, Y. Oyori, and H. Tanazawa, *J. Bioact. Compt. Polym.*, 4, 323 (1989) which are hereby incorporated). However, application of this process is presently limited to surfaces that tolerate water-free organic solvents. With the water insensitive compound I, one can first link I to the surface in water (thus avoiding water-free organics that will damage many surfaces) through one reactive group at one end of the PEG chain, and then subsequently couple a molecule to the remaining reactive aldehyde group at the other end of the PEG chain, as shown in Scheme I. There is no loss of activity of the "remaining aldehyde group" during the first step as there would be with currently available active PEGs. More typical activated PEGs cannot be used in this fashion since they react with water too rapidly to permit the two-step process.

Scheme I

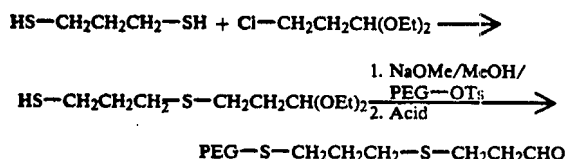


We will not go into the potential applications for this linking technology, but suffice it to say that many exist (see for example articles by M. B. Stark, J. M. Harris, mentioned above and also: H. A. Jacobs, T. Okano, and S. W. Kim, *J. Biomed. Mat. Res.*, 23, 611 (1989); and S. Nagaoka, H. Kurumatani, Y. Mori, and H. Tanazawa, *J. Bioact. Compt. Polym.*, 4, 323 (1989) which are hereby incorporated by reference.

Less important than water stability, but still of significance are two other chemical properties of the aldehyde. First the aldehyde reacts only with amines and this gives desired selectivity. Second, reduction of the first-formed imine intermediate gives a stable amine coupling linkage that could be of advantage relative to previously available linkages, for long term applications.

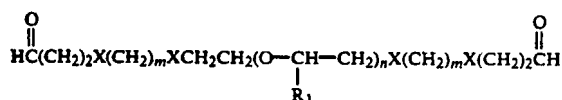
PREPARATION OF PEG PROPIONALDEHYDE OR EQUIVALENTS

To obtain a reactive PEG aldehyde, which is resistant to aldol decomposition, we have prepared PEG-propionaldehyde (PEG—CH₂CH₂CHO, I). The compound can be prepared by reaction of the diethyl acetal of 3-chloropropionaldehyde with PEG alkoxide followed by hydrolysis (yield about 50%). A more effective route to the chemically equivalent sulfur analog is as follows (in DMSO):



Proton NMR is useful for identifying compound I. The intermediate acetal gives the following spectrum (in DMSO): 1.11 ppm, 6H, t, methyls; 1.77 ppm, 4H, m, —CH₂—CH₂—CH₂—; 2.54 ppm, m, 6H, —CH₂S—; 3.50 ppm, —O—CH₂—; 4.55 ppm, t, 1H, acetal CH. And the final product gives the spectrum (in DMSO): 1.78 ppm, t, 2H, —CH₂—CH₂—CH₂—; 2.54 ppm, 8H, m, CH₂S, 2.73 ppm, t, 2H, CH₂—CHO; 3.51 ppm, m, —O—CH₂—, backbone, 9.64 ppm, 1H, s, —CHO.

It should be noted that many other syntheses of compound I can be devised. The presence of sulfur atoms in the prepared analog of I is not of chemical importance; i.e., no observed chemical difference exists between the compounds with sulfur substituted for oxygen in the chain. Similarly, it is to be expected that many related PEG aldehydes can be devised which will prove chemically equivalent. For instance, compounds which are hetero-substituted with Se, Te, N, or P at the O site, and those with a hydrocarbon group attached to the carbon adjacent to the oxygen site, should be chemically equivalent. Compounds with the general formula:



where R₁ is H or a C₁ to C₆ alkyl, and X is O, S, Se, Te, or NR₂ where R₂ is H or a C₁ to C₆ alkyl, should be chemically equivalent to PEG propionaldehyde.

In sharp contrast to PEG-acetaldehyde, compound I is stable in the presence of mild base, and, in contrast to PEG-benzaldehyde, compound I is highly reactive in forming the imine from methyl amine. Reaction of I with methylamine in DMSO at room temperature is complete (as shown by proton NMR) within 5 minutes. Imine formation is reversible in this case, and sodium cyanoborohydride should be added to give reductive amination.

Compound I is ideal for protein modification. Reductive amination proceeds rapidly under a variety of conditions. In a typical preparation we react about three mg of I for every mg of protein, along with a slight excess of cyanoborohydride, for one hour at room temperature in pH 9 borate buffer, followed by dialysis against phosphate-buffered saline; high pH is utilized to avoid reduction of aldehyde. Under these conditions we find that 30–40% of the available lysines are modified, and we find that partitioning of the modified protein in a

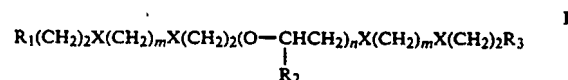
two-phase system is dramatically shifted. Two examples follow. The antibody against alkaline phosphatase (anti-alkaline phosphatase) is 38% modified under these conditions, and the modified protein partitions (in a system consisting of 8% dextran T-40, 6% PEG 8000, 0.15 M NaCl, 0.010 M sodium phosphate, pH 7.2) 99% to the top phase; unmodified protein partitions 90% to the bottom phase. Similarly, the antibody against human red blood cells (anti-human RBCs) is 28% modified under these conditions, and the modified protein partitions (in a system consisting of 4.55% dextran T-500, 3.86% PEG 8000, 0.15 M NaCl, 0.010M sodium phosphate, pH 7.2) 43% to the top phase; unmodified protein partitions almost entirely to the bottom phase. Compound I readily attaches to glass and polymethylmethacrylate, which have been aminated. These same surfaces can then effectively bind proteins.

So in summary, PEG-propionaldehyde I is much more reactive toward amines than PEG-benzaldehyde, stable in water, stable to base, and reactive with lysine groups of proteins (i.e., reductive amination).

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

What is claimed as new and desired to be secured by Letters Patent of the United States is:

1. A compound, as described by formula (I):



wherein R₁ and R₃ each independently consist of an element selected from the group consisting of



R₄—N=CH, and R₄—NH—CH₂, wherein R₄ is an antibody; and wherein X is selected from elements of the group consisting of O, and S; and wherein R₂ is a C₁ to C₆ alkyl, or H, and;

wherein n is an integer, and n less than 10,000, and m is an integer selected from the group consisting of 2, 3, 4, 5, or 6.

2. A compound, as recited in claim 1, wherein R₁ and R₃ are



X is an element selected from the group consisting of O and S, and R₂ is H, m=2 or 3.

3. A composition comprising a compound as recited in any of claims 1 or 2, further comprising water.

4. A compound, as recited in any one of claims 1, or 2, wherein n is between 10 and 1,000.

5. A process for amine modifications comprising the steps of:

preparing an amine comprising substance in a solution and adding a compound, I,

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IN BLOOD AND PROCESS FOR THE PREPARATION THEREOF

1 2 3 4

(57) Abstract: A conjugate of a hG-CSF derivative modified with a PEG shows an en-
hanced *in vivo* stability and prolonged half-life in blood, while reducing the possibility of
inducing an immune response.

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HUMAN GRANULOCYTE-COLONY STIMULATING FACTOR CONJUGATE HAVING ENHANCED STABILITY IN BLOOD AND PROCESS FOR THE PREPARATION THEREOF

5 Field of the Invention

The present invention relates to a conjugate of a human granulocyte-colony stimulating factor (hG-CSF) derivative modified with a polyethylene glycol (PEG) and a method for the preparation thereof.

10

Background of the Invention

Granulocyte-colony stimulating factors (G-CSFs) are cytokines that stimulate the expansion and differentiation of bone marrow progenitor cells and
15 leukocytes. They also activate mature myeloid cells to mediate a variety of anti-microbial activities and inflammatory responses. They have been widely used in clinical transplantation which induces hematopoietic stem cell mobilization and increases myeloid progenitor cell population. It has been reported that the purified G-CSF is highly active in inducing granulocyte
20 differentiation and suppresses stem cell self-replication in myeloid leukemia cells (Nicola et. al., *JBC* 258(14): 9017-9023, 1983). Recently, recombinant G-CSF proteins, sold under the brand names of Filgrastim and Neulasta, have been increasingly employed in the treatment of various diseases including cancer and hematopoietic disorders.

25 Human G-CSF is a glycoprotein, consisting of 174 amino acids, which plays an important role in hematopoietic cell proliferation, differentiation of hematopoietic precursor cells, and activation of mature neutrophilic granulocytes. hG-CSF gene transcribes into two kinds of mRNA consisting of 207 and 204 amino acids, respectively, that contain a leader sequence consisting
30 of 30 amino acids for their secretion at the terminal amino residue (Nagata et al.,

Nature 319: 415-418, 1986; Nagata et al., *EMBO J.* 5(3): 575-581, 1986; Souza et al., *Science* 232: 61-65, 1986). The difference in the two hG-CSFs is the presence or absence of three amino acids (Val-Ser-Glu) at the 35th amino acid residue starting from the terminal amino residue. Accordingly, a mature hG-
5 CSF protein has 174 or 177 amino acids. hG-CSF consisting of 174 amino acids shows a 20-fold higher activity than hG-CSF consisting of 177 amino acids. However, whether both hG-CSFs are actually expressed *in vivo* is not elucidated at this time. hG-CSF does not contain the amino acid sequences (Asn-X-Ser/Thr) relating to N-glycosylation, but has an O-glycosylation site at
10 the 133rd amino acid residue, threonine.

In order to purify G-CSF, a method which comprises culturing a G-CSF producing cell and extracting G-CSF from a culture supernatant has been widely employed; however, the method suffers from various problems associated with the low yield of G-CSF and complicated purification process. It has been
15 reported that the amino acid sequence of hG-CSF and a gene encoding the same can be established by using the existing methods (Korean Patent Laid-Open Publication Nos. 91-5624 and 92-2312), and a method for preparing a substance showing hG-CSF activity by using genetic engineering is developed (Korean Patent Nos. 47178, 53723 and 57582). In the previously reported methods, a
20 glycosylated hG-CSF glycoprotein is prepared from mammalian cells using a genomic DNA or cDNA encoding hG-CSF. The glycosylated hG-CSF thus produced has O-glycosidic type sugar chain structures, but it has been known that the sugar moiety is not essential for hG-CSF activity (Lawrence, M. et al., *Science* 232: 61, 1986). Further, since these methods need to employ an
25 expensive culture medium and complicated purification process to obtain the glycosylated hG-CSF, they are uneconomical.

Further, widely employed in a method for producing non-glycosylated hG-CSF using a prokaryotic cell is a process using *E. coli* which produces hG-CSF consisting of 175 or 178 amino acids according to the characteristics of *E.*
30 *coli* cell. These products have an extra methionine residue added at the

terminal amino residue by the action of the ATG codon existing at the site of initiation codon. However, it has been reported that the additional methionine can trigger harmful immune response (EP Patent Publication No. 256,843). Furthermore, most of the expressed hG-CSF accumulates in cytoplasm in the form of insoluble inclusion bodies and must be converted into an active form through refolding during purification. Such a refolding process is not efficient, and therefore, hG-CSF exists partially in a reduced form, or forms an intermolecular disulfide coupling body or a defective disulfide coupling body. It is difficult to remove these by-products, which markedly lower the yield. In particular, it is extremely difficult to remove undesirable hG-CSF by-products such as misfolded hG-CSF. In addition, hG-CSF has five cysteine residues, four of which participate in disulfide bonding, while the remaining cysteine in the unbound free form reduces the folding titer and stability in solution. Further, non-glycosylated hG-CSF expressed in *E. coli* is apt to aggregate at a high concentration because of its low solubility.

To solve these problems, the present inventors have endeavored to develop a method for producing a soluble hG-CSF having no methionine residue added at the terminal amino residue thereof by using a microorganism. The present inventors have previously generated a new signal peptide of *E. coli* thermostable enterotoxin II (Korean Patent Application No. 98-38061) and found that this new secretory signal peptide can be used for the mass-production of the native form of hG-CSF. Further, the present inventors have found that when an hG-CSF derivative obtained by replacing the 17th cysteine residue with other amino acid ligated to the modified *E. coli* thermostable enterotoxin II signal sequence is expressed, hG-CSF is produced in a wild-type form having no methionine residue added at its terminal amino residue in the periplasm of *E. coli* (Korean Patent No. 356140).

Meanwhile, the problem of using a peptide drug *in vivo* is that its half-life is too low to exert a satisfactory therapeutic effect. Accordingly, there have been several attempts to develop a peptide drug having an enhanced half-

life in blood to reduce the injection interval so that a high pharmacological efficacy and quality of patient's life can be maintained. One of the most widely used methods for improving the stability of a protein is pegylation, which involves chemical modification of a polypeptide with a highly soluble moiety such as polyethylene glycol (PEG). When linked to a peptide drug specifically or non-specifically, PEG increases the solubility of the peptide drug to water or an organic solvent, reduces the immune response and enhances *in vivo* stability. Further, when a protein has a small molecular weight, it may be lost in the process of clearing intestinal tract, kidney, spleen or liver through filtration, but such pegylation may reduce the clearance loss (Knauf, M. J. et al, *J. Biol. Chem.* 263: 15064, 1988). Many studies to prolong the *in vivo* half-life, increase the solubility or reduce the immune response by coupling PEG to a biologically active peptide or a protein have been actively progressed. It has been well known that, pegylation increases the solubility of peptide drug and prevents the hydrolysis thereof, thereby increasing the serum stability of the peptide drug without incurring any immune response due to its low antigenicity (Sade et al., *J. Fermentation Bioengineering* 71: 1370139, 1991). However, such pegylated polypeptides have the disadvantages of lowering both the activity and production yield of an active substance when the molecular weight of PEG increases.

Usually, PEG is covalently linked to one or more of free lysine residues existing at the surface of a protein, but if PEG binds to an active site directly involved in protein activity, the protein activity decreases. Further, since the binding of PEG to a lysine residue occurs in a random fashion, many kinds of pegylated protein complexes may co-exist as a mixture, and therefore, a purification process of a desired complex becomes more complicated and difficult (EP Patent Publication Nos: 0401384 and 0335423). To solve such problems, US Patent No: 5,766,897 and International Patent Publication No: WO 00/42175 teach that PEG-maleimide can be used for the selective binding of PEG to a cysteine residue to prevent PEG from interacting with an active site

of human growth hormone (hGH) in a coupling reaction of hGH to PEG. However, this coupling reaction demands at least one free cysteine residue which is not engaged in a disulfide bonding, but all four cysteine residues of hGH are involved in the formation of disulfide bonding. Accordingly, the method described above performs a pegylation reaction using a hGH derivative having an extra cysteine residue added by insertion. However, several problems have been reported that the protein employed in the pegylation reaction is not a wild-type and it is very difficult to express and purify the protein having an additional free cysteine residue. EP Patent Publication No: 0733067 has disclosed a method for preparing a uniform G-CSF composition which comprises a PEG-G-CSF complex prepared by a selective coupling of PEG to a terminal amino residue of G-CSF. A fusion protein of PEG-G-CSF complex thus prepared shows enhanced stability, but this method does not use a wild-type G-CSF and produces an inactive protein having an extra methionine residue added at the terminal amino residue in the form of an inclusion body. Thus, they have the problems of triggering harmful immune response, requiring a refolding process and a markedly low yield.

To solve the mentioned problems, the present inventors have developed a PEG conjugate of an hG-CSF derivative having the 17th amino acid of the wild-type hG-CSF replaced with serine without an extra methionine residue added at the terminal amino residue. The PEG-hG-CSF derivative conjugate of the present invention has a prolonged *in vivo* half-life without inducing an immune response in a subject, while minimizing the biological activity loss.

Summary of the Invention

Accordingly, a primary object of the present invention is to provide a PEG conjugate of an hG-CSF derivative having a prolonged *in vivo* half-life and an improved biological activity without inducing an immune response in a subject; and a method for the preparation thereof.

15

Another object of the present invention is to provide a pharmaceutical composition comprising said PEG conjugate of hG-CSF derivative.

A further object of the present invention is to provide a method for enhancing the *in vivo* stability and prolonging the circulating half-life of hG-CSF, without sacrificing the activity thereof.

In accordance with one aspect of the present invention, there is provided a PEG-hG-CSF derivative conjugate having a prolonged *in vivo* half-life which is prepared by modifying the terminal amino residue of hG-CSF derivative with one PEG molecule; and a method for the preparation thereof.

In accordance with another aspect of the present invention, there is provided a pharmaceutical composition of hG-CSF having a prolonged *in vivo* half-life in comparison with unmodified hG-CSF, which comprises said PEG-hG-CSF derivative conjugate and a pharmaceutically acceptable carrier.

In accordance with still another aspect of the present invention, there is provided a method for enhancing the *in vivo* half-life of hG-CSF, which comprises specifically modifying the terminal amino residue of hG-CSF derivative with one PEG molecule.

Brief Description of the Drawings

The above and other objects and features of the present invention will become apparent from the following description of the invention, when taken in conjunction with the accompanying drawings which respectively show:

Fig. 1: SDS-PAGE results of PEG-hG-CSF derivative conjugates;

Fig. 2: a size exclusion chromatogram of PEG-hG-CSF derivative conjugates;

Fig. 3: a result for comparing the peptide map of a PEG-hG-CSF derivative conjugate (B) with that of unmodified hG-CSF (A);

Fig. 4: *in vivo* activities of PEG-hG-CSF derivative conjugates; and

Fig. 5: a pharmacokinetic graph showing the enhanced circulating half-life of a PEG-hG-CSF derivative conjugate.

Detailed Description of the Invention

5

The present invention relates to a conjugate of human G-CSF derivative (Korean Patent No: 356140) modified with PEG and a method for the preparation thereof.

10 The PEG conjugate of an hG-CSF derivative is a protein conjugate prepared by selectively modifying the terminal amino residue of an hG-CSF derivative with PEG, wherein the terminal amino residue does not necessarily participate in the binding of hG-CSF to a receptor thereof (Hill et, al., *PNAS* 90: 5167, 1993).

15 The hG-CSF derivative employed in the present invention may be either a natural one isolated from a mammal or a chemically synthesized recombinant. Further, it may also be obtained from prokaryotic or eukaryotic cells transformed with DNA coding the hG-CSF derivative by genetic engineering. For this purpose, *E. coli*, yeast (e.g., *S. cerevisias*) and mammalian cells (e.g., Chinese hamster ovary cell, CHO) can be employed as a host. According to
20 the characteristics of host cell used, the protein product expressed from hG-CSF derivative-transformed mammalian cells or eukaryotic cells may be glycosylated or non-glycosylated with a host cell-derived carbohydrate. Further, in case of expressing from hG-CSF derivative-transformed prokaryotic cells, the protein product may include an incipient methionine residue at -1
25 position starting from the translation initiation codon.

In a preferred embodiment of the present invention, a recombinant hG-CSF derivative obtained by replacing the 17th amino acid residue of wild-type hG-CSF with serine according to the method described in Korean Patent No. 356140.

30 PEG employable in the present invention may be any one of known

soluble PEG polymers. In the process of modifying a protein with a PEG polymer having a reactive group, the PEG binding occurs at the ϵ -amino group of lysine, cysteine or histidine residue depending on the nature of the reactive group of PEG. But, when such a PEG polymer having a reactive group is used, two more of PEG molecules may bind to a hG-CSF derivative, leading to the loss of its protein activity.

Accordingly, the binding between the hG-CSF derivative and PEG preferably occurs in a molar ratio of 1:1, and a suitable PEG polymer for this purpose is soluble in an aqueous environment such as a physiological environment and has a single reactive aldehyde group which specifically binds to the α -amino group of the terminal amino residue of hG-CSF derivative. Preferably, such a PEG has the aldehyde reactive group at one end and an alkoxy group at the other end, and the terminal amino residue is threonine. Further, the molecular weight of said PEG is preferably, but not to be limited to, in the range of 2 to 100 kDa, more preferably, 10 to 40 kDa; and said PEG may be branched or not.

To couple a PEG to an hG-CSF derivative, a pegylation reaction involving the amino group of the hG-CSF derivative and the aldehyde group of the PEG is carried out in the presence of a reducing agent. Preferably, the alkoxy-PEG-aldehyde is methoxy-PEG-aldehyde, and the reducing agent is cyanoborohydride (NaCNBH_3), sodium borohydride, dimethylamine borate or pyridine borate. Further, it is preferable to employ the hG-CSF derivative and PEG in a molar ratio ranging from 1:1 to 1:50 in the pegylation reaction. After the pegylation reaction is completed, whether a PEG-G-CSF derivative conjugate is generated or not can be examined by SDS-PAGE. Subsequently, a fraction containing the desired PEG-hG-CSF derivative conjugate may be isolated utilizing the difference in the appearance charge by using cation exchange chromatography. The resulting fraction thus obtained is subjected to size exclusion chromatography to purify the PEG-hG-CSF derivative conjugate.

To examine whether PEG is specifically linked to the terminal amino

residue of hG-CSF derivative, the peptide map of PEG-hG-CSF derivative conjugate is drawn and compared with the previously reported peptide map of hG-CSF derivative (*Journal of chromatography A*, 808: 121, 1998; *Analytical Biochemistry* 122: 348, 1982; and *Biotechnology and Applied Biochemistry* 10: 326, 1988). Namely, when the PEG-hG-CSF derivative conjugate and unmodified hG-CSF are subjected to protease cleavage, the HPLC peak pattern of PEG-hG-CSF derivative conjugate differs from that of unmodified hG-CSF in specific ways due to the binding of PEG to one of protease cleavage fragments of PEG conjugate. The protease may be, but not limited to, trypsin, subtilisin or endoproteinase Glu-C; and, more preferably, endoproteinase Glu-C.

The biological activity of the inventive PEG-hG-CSF derivative conjugate is measured according to the conventional methods well-known in the art (Baldwin et al., *Acta Endocrinologica*. 119: 326, 1988; Clark et al., *J. Biol. Chem.* 271(36): 21969, 1996; and Bozzola et al., *J. endocrinol. Invest.* 21: 768, 1998). As a result, the PEG-hG-CSF derivative conjugate shows a prolonged *in vivo* half-life, enhanced biological activity, and reduced toxicity and immunogenicity.

Accordingly, the present invention provides a pharmaceutical composition of hG-CSF having a prolonged *in vivo* half-life, which comprises the inventive PEG-hG-CSF derivative conjugate and a pharmaceutically acceptable carrier.

The pharmaceutical composition of the present invention can be administered via various routes including oral, transdermal, subcutaneous, intravenous and intramuscular introduction, and injection is more preferred. The composition of the invention may be formulated so as to provide quick, sustained or delayed release of the active ingredient after it is administered to a patient, by employing any one of the procedures well-known in the art. The formulation may be in the form of a tablet, pill, powder, sachet, elixer, suspension, emulsion, solution, syrup, aerosol, soft and hard gelatin capsule, sterile injectable solution, sterile packaged powder and the like. Examples of

12

suitable carriers, excipients or diluents are lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, alginates, gelatin, calcium phosphate, calcium silicate, polyvinylpyrrolidone, cellulose, methylcellulose, microcrystalline magnesium stearate and mineral oil. The formulation may additionally include
5 fillers, anti-agglutinating agents, lubricating agents, wetting agents, flavoring agents, emulsifiers, preservatives and the like.

The amount of the active ingredient actually administered ought to be determined in light of various relevant factors including the condition to be treated, the chosen route of administration, the age, sex and body weight of the
10 individual patient, and the severity of the patient's syndrome, especially, the kind of active ingredient. Preferably, a typical daily dose of the inventive PEG-hG-CSF derivative conjugate as an effective ingredient may range from about 0.01 to 100 $\mu\text{g/kg}$ body weight (calculating the mass of protein alone, without chemical modification). Owing to the enhanced stability of PEG-hG-CSF
15 derivative conjugate of the invention, the total number and frequency of the administration of the formulation comprising the inventive PEG-G-CSF derivative conjugate can be considerably reduced.

The present invention is further defined in the following Examples. It
20 should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention
25 to adapt it to various usage and conditions.

Example 1: Preparation of recombinant human G-CSF derivative

Recombinant hG-CSF derivative, HM10411 was prepared according to
30 the method described in Korea Patent No: 356140. The hG-CSF derivative

thus prepared has the 17th amino acid of wild-type G-CSF replaced with serine (¹⁷S-G-CSF).

Example 2: Preparation of PEG-hG-CSF derivative conjugate

5 HM10411 was dissolved in 100 mM potassium phosphate buffer (pH 6.0) to a concentration of 5 mg/ml, distributed into three vials, and then, adjusted to a final volume of 5 ml. An activated methoxy-PEG-aldehyde (Shearwater, USA) which has 20 kDa or 40 kDa of PEG was added to each of
10 two vials in an amount corresponding to a HM10411:methoxy-PEG-aldehyde molar ratio of 1:4. Sodium cyanoborohydride (NaCNBH₃, Sigma, USA) was added thereto to a final concentration of 20 mM, and the reaction mixture was stirred at 4°C for 18 hrs. Then, ethanolamine was added thereto to a final concentration of 50 mM to inactivate unreacted PEG.

15 To further remove unreacted PEG, the reaction mixture was subjected to Sephadex G-25 column chromatography (Pharmacia). Namely, after the column was equilibrated with 2 CV (column volume) of 10 mM Tris-HCl buffer (pH 7.5), the reaction mixture was loaded onto the column and eluted with the same buffer. Elution fractions were analyzed for absorbance at 260 nm using
20 a UV spectrophotometer. As a result, the PEG modified HM10411 which has a large molecular weight was eluted before unreacted PEG.

Example 3: Isolation of mono-PEG-hG-CSF derivative conjugate

25 The PEG modified HM10411 was further purified from the elution fraction obtained in Example 2 as follows.

A column packed with 3 ml of PolyWAX LP (Polywax Inc., USA) was equilibrated with 10 mM Tris-HCl buffer (pH 7.5). The elution fraction containing the PEG modified HM10411 was loaded onto the column at a flow
30 rate of 1 ml/min, and the column was washed with 5 CV (15 ml) equilibration

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(54) Title: PHARMACEUTICAL COMPOSITIONS WITH ENHANCED STABILITY

(57) Abstract: The present invention provides for a stabilized biodegradable polymeric composition useful as a controlled release delivery system for peptide agents. The compositions of the present invention comprise a) a beneficial salt of a peptide agent formed with a strong acid that minimizes or prevents the interaction/reaction between the peptide agent and the polymer in an organic solution; b) a biodegradable polymer; c) a pharmaceutically acceptable organic solvent; and d) optionally one or more excipients. The present invention also relates to a method of manufacturing and a method of use thereof.

5 significant loss of activity or change of immunogenicity. The molecular weight of the polymer also decreased significantly within the same time period. This fast degradation of the peptide and polymer will alter the release profile of the peptide and result
10 in a compromised treatment outcome. Therefore, precise control for the preparation process and time is critical and this significantly increases the difficulty for end-user. Furthermore, the *in vivo* formation of the implant from the injectable polymeric composition is not
15 instantaneous. Typically the solvent dissipation process can take a few hours to several days depending upon the solvents used. During this period, the presence of an organic solvent could also promote the interaction/reaction between the peptide agents and the
20 polymer. Therefore, there is a need to develop a pharmaceutical composition that will minimize or prevent the interaction/reaction between the peptide agent and the polymer in an organic solution. There is a further need to develop a pharmaceutical composition that is
25 stable with a satisfactory storage shelf life in a ready-to-use product configuration.

SUMMARY OF THE INVENTION

[0007] It was surprisingly discovered that injectable
30 biodegradable polymeric compositions comprising peptide agents in the form of a salt formed with a strong acid (e.g., hydrochloric acid) exhibit much higher stability than those in the form of a salt formed with a weak acid (e.g., acetic acid) or in the form of the free base.
35 Such beneficial salts of peptide agents may be formed through the neutralization of any basic groups of the

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5 peptide agents with a strong acid. When such beneficial
salts of peptide agents formed with a strong acid were
formulated into injectable biodegradable polymeric
compositions, the interactions/reactions between the
peptide agents and the polymer are minimized or
10 prevented. Using such beneficial salts of peptide
agents formed with a strong acid allows for the
preparation of a stabilized injectable composition pre-
filled in a single syringe in a ready-to-use
configuration with satisfactory storage stability. The
15 use of the salt of peptide agent formed with a strong
acid of the present invention to enhance the stability
of the injectable polymeric compositions is not
contemplated by the prior art.

[0008] Accordingly, the present invention provides
20 a stabilized injectable biodegradable polymeric
composition for forming an economical, practical, and
efficient controlled release delivery system for peptide
agents. The present invention also provides a method of
manufacturing and a method of use thereof. According to
25 the present invention, the drug delivery system is
produced easily and delivered conveniently to a subject
such as a mammal or human. The compositions deliver
therapeutic amount of peptide agents over a desired,
extended period of time, preferably from several weeks
30 to one year. The compositions are both biocompatible
and biodegradable, and disappear harmlessly after
delivering the dose of the peptide agents.

[0009] The compositions in accordance with the
present invention comprise a) a beneficial salt of a
35 peptide agent formed with a strong acid that minimizes
or prevents the interaction/reaction between the peptide

5 agent and the polymer in an organic solution; b) a biodegradable polymer; c) a pharmaceutically acceptable organic solvent. According to the invention, the pharmaceutical composition may optionally include excipients to achieve optimal delivery of the peptide.
10 agent. The pharmaceutical composition may be a viscous or non-viscous liquid, gel or semisolid that moves as a fluid so that it may be injected using a syringe. The pharmaceutical composition may be pre-filled into one syringe to form a product in a ready-to-use
15 configuration.

[0010] The peptide agent of the present invention contains at least one basic group. The peptide agent may be any peptide, oligopeptide, polypeptide, or protein that is capable of providing a biological,
20 physiological or therapeutic effect in an animal or human. The peptide agent may be any one or more of known biologically active peptide, oligopeptide, polypeptide, or protein recognized in any documents cited herein or otherwise recognized in the art. The peptide agent may
25 also stimulate or inhibit a desired biological or physiological activity within the animal or human, including without limitation, stimulate an immunogenic or immunological response.

[0011] According to one embodiment of the present
30 invention, the peptide agent has an N-terminus that is not a primary amine (e.g., LHRH agonists, such as leuprorelin, goserelin, LHRH antagonists, such as cetrorelix, enfuvirtide, thymosin α 1, abarelix, and the like). In another embodiment of the present invention,
35 the peptide agent has either an N-terminal primary amine or side chain primary amine group covalently modified

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5 with hydrophilic and/or lipophilic moieties that may be produced through pegylation, acylation, and the like. Furthermore, both N-terminal primary amine and side chain primary amine groups of the peptide agent may also be covalently modified simultaneously with hydrophilic
10 and/or lipophilic moieties through pegylation, acylation, and the like.

[0012] The strong acid may be any acid having a pKa in water less than 3, preferably less than 0, more preferably less than -3. For example, a strong acid may
15 be selected from, but not limited to, the group consisting of hydrochloric acid, hydrobromic acid, sulfuric acid, organic sulfuric acids, alkyl sulfuric acids of 1-40 carbons, nitric acid, chromic acid, methanesulfonic acid, trifluoromethane sulfonic acid,
20 organic sulfonic acids, trichloroacetic acid, dichloroacetic acid, bromoacetic acid, chloroacetic acid, cyanoacetic acid, 2-chloropropanoic acid, 2-oxobutanoic acid, 2-chlorobutanoic acid, 4-cyanobutanoic acid, perchloric acid, phosphoric acid, hydrogen iodide,
25 and the like.

[0013] The biodegradable polymer can be any biocompatible and pharmaceutically acceptable polymers. The biodegradable polymers may be thermoplastic, which melts upon heating and solidifies upon cooling. The
30 biodegradable polymers of the invention are substantially insoluble in aqueous or body fluid, but are capable of substantially dissolving or dispersing in a water-miscible organic solvent to form a solution or suspension. Upon contact with an aqueous fluid, the
35 water-miscible organic solvent diffuses/dissipates from the inventive composition, which causes the coagulation

5 of the polymer to form a gel, or solid matrix encapsulating the peptide agent. Examples of the polymers suitable for the present composition includes, without limitation, polylactides, polyglycolides, polycaprolactones, polyanhydrides, polyurethanes, 10 polyesteramides, polyorthoesters, polydioxanones, polyacetals, polyketals, polycarbonates, polyorthocarbonates, polyphosphazenes, polyhydroxybutyrates, polyhydroxyvalerates, polyalkylene oxalates, polyalkylene succinates, poly(malic acid), 15 poly(maleic anhydride), and copolymers, terpolymers, or combinations or mixtures therein. Lactic acid-based polymers, and copolymers of lactic acid and glycolic acid (PLGA), including poly(D,L-lactide-co-glycolide) and poly(L-lactide-co-glycolide) are preferably used in 20 the present invention. In some embodiments, the PLGA polymers have a weight average molecular weights of between about 2,000 to about 100,000 and monomer ratios of lactic acid to glycolic acid of between about 50:50 to about 100:0.

25 [0014] The pharmaceutically acceptable organic solvents may be selected from a group consisting of N-methyl-2-pyrrolidone, methoxypolyethylene glycol, alkoxypolyethylene glycol, polyethylene glycol esters, glycofurol, glycerol formal, methyl acetate, ethyl 30 acetate, methyl ethyl ketone, dimethylformamide, dimethyl sulfoxide, tetrahydrofuran, caprolactam, decylmethylsulfoxide, benzyl benzoate, ethyl benzoate, triacetin, diacetin, tributyrin, triethyl citrate, tributyl citrate, acetyl triethyl citrate, acetyl 35 tributyl citrate, triethylglycerides, triethyl phosphate, diethyl phthalate, diethyl tartrate, ethyl

5 lactate, propylene carbonate, ethylene carbonate, butyrolactone, and 1-dodecylazacyclo-heptan-2-one, and combinations thereof.

[0015] According to the present invention, one or more excipients may be incorporated in the inventive
10 composition to achieve optimal delivery of the peptide agent. Suitable excipients may include release rate modifying agents, burst effect reducing materials, buffering materials, antioxidants, and the like.

[0016] According to the present invention, suitable
15 release rate modifying agents include, but are not limited to, amphiphilic compounds or copolymers, such as alkanecarboxylic acid, oleic acid, alkyl alcohol, polar lipids, surfactants, copolymers of polyethyleneglycol and polylactide or poly(lactide-co-glycolide),
20 poloxamers, polyvinylpyrrolidone, polysorbates, and the like; esters of mono-, di-, and tricarboxylic acids, such as 2-ethoxyethyl acetate, triethyl citrate, acetyl tributyl citrate, acetyl triethyl citrate, glycerol triacetate, di(n-butyl) sebecate, and the like;
25 polyhydroxy alcohols, such as polyethylene glycol, sorbitol, and the like; fatty acids; triesters of glycerol, such as triglycerides, medium-chain triglycerides such as MIGLYOL 810, 812, 818, 829, 840, and the like. Mixtures of rate modifying agents can also
30 be used in the polymer systems of the invention.

[0017] According to the present invention, suitable buffering agents include, but are not limited to, inorganic and organic salts including calcium carbonate, calcium hydroxide, calcium myristate; calcium oleate,
35 calcium palmitate, calcium stearate, calcium phosphate, magnesium carbonate, magnesium hydroxide, magnesium

5 phosphate, magnesium myristate, magnesium oleate, magnesium palmitate, magnesium stearate, zinc carbonate, zinc hydroxide, zinc myristate, zinc oleate, zinc palmitate, zinc stearate, zinc phosphate, and combinations thereof.

10 [0018] According to the present invention, suitable antioxidants include, but are not limited to, d-alpha tocopherol acetate, ascorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, butylated hydroxyquinone, hydroxycomarin, butylated
15 hydroxytoluene, ethyl gallate, propyl gallate, octyl gallate, lauryl gallate, propylhydroxybenzoate, trihydroxybutylphenone, vitamin E, pegylated vitamin E or vitamin E-TPGS, and the like.

[0019] The present invention further provides methods
20 of making and using such compositions. For example, a method of making such compositions comprising the neutralization of basic amine groups of peptide agents to form a beneficial salt to minimize or prevent the interaction/reaction of the basic amine group with the
25 polymer; and the combination of the beneficial salt with other components and optionally one or more excipients. Preferably, the beneficial salt of the peptide agent is formed first, and then combined with the polymer dissolved in an organic solvent. Such compositions are
30 physico-chemically stable prior to and during the fabrication process of a controlled delivery system such as microparticle formation or other implantable matrix formation. Preferably, such injectable compositions are physico-chemically stable during preparation, storage,
35 and subsequent administration to a subject and form

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5 consistent and controlled release implants upon administration to a tissue site.

[0020] The present invention further provides a kit for administration of the injectable composition to form a consistent and controlled release depot system, the
10 kit comprises: a biodegradable polymer dissolved in a pharmaceutically acceptable solvent; a beneficial salt of a peptide agent containing at least one basic amine group formed with a strong acid dissolved or dispersed in the polymeric vehicle; and optionally one or more
15 excipients. The uniform mixture of all the components is packaged into one container. Preferably, the container is a syringe. Accordingly, the present invention also provides a method comprising a step of filling a syringe with the composition to form a stable product in a
20 ready-to-use configuration.

[0021] The present invention further provides a method for *in-situ* forming implant capable of functioning as a controlled release delivery system of the peptide agent in a subject. The peptide agent is
25 preferably incorporated into the *in situ* formed implant, and subsequently released into the surrounding tissue fluids and to the pertinent body tissue or organ as the polymer degrades. The method comprises: administration of the injectable compositions of the present invention
30 to an implant site by any suitable method for applying a liquid, as for example, by means of a syringe, needle, cannula, catheter, pressure applicator, and the like.

BRIEF DESCRIPTION OF THE FIGURES

35 [0022] Figure 1. Stability of LA in Formulations at 4 °C after 16 Months

5 Peptide agents useful in the preparation of the
compositions of the present invention include, but are
not limited to, oxytocin, vasopressin,
adrenocorticotrophic hormone (ACTH), epidermal growth
factor (EGF), platelet-derived growth factor (PDGF),
10 prolactin, luteinising hormone, luteinizing hormone
releasing hormone (LHRH), LHRH agonists, LHRH
antagonists, growth hormones (including human, porcine,
and bovine), growth hormone releasing factor, insulin,
erythropoietin (including all proteins with
15 erythropoietic activity), somatostatin, glucagon,
interleukin (which includes IL-2, IL-11, IL-12, etc.),
interferon-alpha, interferon-beta, interferon-gamma,
gastrin, tetragastrin, pentagastrin, urogastrone,
secretin, calcitonin, enkephalins, endorphins,
20 angiotensins, thyrotropin releasing hormone (TRH), tumor
necrosis factor (TNF), parathyroid hormone (PTH), nerve
growth factor (NGF), granulocyte-colony stimulating
factor (G-CSF), granulocyte macrophage-colony
stimulating factor (GM-CSF), macrophage-colony
25 stimulating factor (M-CSF), heparinase, vascular
endothelial growth factor (VEG-F), bone morphogenic
protein (BMP), hANP, glucagon-like peptide (GLP-1),
exenatide, peptide YY (PYY), renin, bradykinin,
bacitracins, polymyxins, colistins, tyrocidine,
30 gramicidins, cyclosporins (which includes synthetic
analogues and pharmacologically active fragments
thereof), enzymes, cytokines, antibodies, vaccines,
antibiotics, antibodies, glycoproteins, follicle
stimulating hormone, kyotorphin, taftsin, thymopoietin,
35 thymosin, thymostimulin, thymic humoral factor, serum
thymic factor, colony stimulating factors, motilin,

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5 bombesin, dinorphin, neurotensin, cerulein, urokinase,
kallikrein, substance P analogues and antagonists,
angiotensin II, blood coagulation factor VII and IX,
gramicidines, melanocyte stimulating hormone, thyroid
hormone releasing hormone, thyroid stimulating hormone,
10 pancreozymin, cholecystokinin, human placental lactogen,
human chorionic gonadotrophin, protein synthesis
stimulating peptide, gastric inhibitory peptide,
vasoactive intestinal peptide, platelet derived growth
factor, and synthetic analogues and modifications and
15 pharmacologically-active fragments thereof.

[0037] The preferred peptide agents used herein
include the peptide agents wherein the N-terminus is not
a primary amine. For example, the N-terminus of the
peptide agents may be a pyroglutamic acid, e.g., LHRH,
20 and LHRH agonists such as leuprorelin, buserelin,
gonadorelin, deslorelin, fertirelin, histrelin,
lutrelin, goserelin, nafarelin, triptorelin, and the
like. Alternatively, the N-terminal amine group may be
capped or acylated, e.g., cetrorelix, enfuvirtide,
25 thymosin α 1, abarelix, and the like.

[0038] The preferred peptide agents used herein also
include the peptide agents wherein the N-terminal
primary amine is covalently modified with hydrophilic
and/or lipophilic moieties such as through pegylation,
30 acylation, and the like. The peptide agents used herein
further include the peptide agents wherein the side
chain primary amine(s) are covalently modified with
hydrophilic and/or lipophilic moieties such as through
pegylation, acylation, and the like. The preferred
35 peptide agents used herein further include the peptide
agents wherein both N-terminal primary amine and side

5 chain primary amine groups are covalently modified simultaneously with hydrophilic and/or lipophilic moieties such as through pegylation, acylation, and the like.

[0039] The term "hydrophilic moiety" refers to any
10 water-soluble linear or branched oligomer or polymer including, but not limited to, polyethylene glycol and polypropylene glycol and similar linear and branched polymers. Preferably, the molecular weight of the polymer ranges from about 500 daltons to about 50,000
15 daltons. Hydrophilic polymers for use in the present invention may have a reactive group incorporated for attachment to the peptide agent of interest through amine, carboxylic, hydroxyl, or thiol groups.

[0040] The term "pegylation" used herein refers to
20 the covalent conjugation of a soluble polyethylene glycol to the peptide agents. Polyethylene glycol can be prepared according to standard protocols with one end capped as with a methoxy group and the other end activated for facile conjugation to active groups on
25 peptide agents. For examples, various methods for preparing polyethylene glycols and their use for pegylations are described in the art: [e.g., Roberts MJ, Bentley MD, Harris JM, Chemistry for peptide and protein PEGylation. Adv Drug Deliv Rev. 2002 Jun 17; 54(4): 459-
30 76. Veronese FM. Peptide and protein PEGylation: a review of problems and solutions. Biomaterials. 2001 Mar; 22(5): 405-17 and US Patents Nos. 6,113,906; 5,446,090; 5,880,255], which are all incorporated herein by reference.

35 [0041] The term "lipophilic moiety" refers to any molecules having a solubility in water at 20 °C less than